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13. ABSTRACT (Maximum 200)  The promise of recent success demonstrating the immunologic recognition of tumor cells has been limited by the paucity of native human tumor specific antigens that might serve as targets for attack. This work focused on a human disorders, paraneoplastic neurologic degeneration (PND), in which breast tumors express tumor specific antigens--proteins normally made only in the nervous system--and come under immunologic attack.  In the first Aim we characterized the expression of PND genes in normal tissue and tumor. We found that cdr2, Nova-1 and Nova-2 are all highly restricted in normal tissues, but are expressed in breast and other tumors. This specificity underlies the antigenicity of these proteins in tumor cells, and provides a potential focus for tumor therapy. In Aims II-III we set out to generate animal models of breast tumor immunity against the cdr2 antigen. In parallel, we recently examined tumor immunity in human PND patients, based on our identification of cdr2 as the tumor antigen in these tumors (Aim 1). These studies allowed us to identify cdr2-specific killer T cells in breast cancer patients, and helped guide our approach to animal models. We have now generated cdr2-specific CTLs in mice inoculated with genetically defined transfected tumor cell lines. These studies allow us to overcome unanticipated difficulties originally encountered in these experiments.				
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## Table of Contents

Page 1:	Front Cover
Page 2:	SF-298
Page 3:	Foreword
Page 4:	Table of Contents
Page 5:	Introduction
Page 6:	Body\
Page 13:	Conclusions
Page 17:	References
Page 19:	Final report bibliography and personnel
Page 20:	Appendix



## INTRODUCTION:

Paraneoplastic neurologic disease (PND) antigens are proteins normally expressed specifically in the central nervous system (CNS) that are expressed ectopically in tumors, most commonly breast, ovarian and lung cancers (Posner and Furneaux, 1990), and we have termed such proteins onconeural antigens (Darnell et al., 1991; Darnell, 1994; Darnell, 1996). It is believed that their expression in tumors outside of the immunologically privileged CNS allows for their recognition by the immune system, and consequently a clinically effective anti-tumor immune response (Posner and Furneaux, 1990; Darnell, 1996). Tumor suppression is a significant factor in the majority of PND patients (Darnell and DeAngelis, 1993; Darnell, 1994; Darnell, 1996).

Studies of the expression and function of onconeural antigens in tumors and brain are lacking, as are animal models of their role in tumor biology and immunology. The purpose of the present work is to expand our understanding of the expression of onconeural antigens, and to develop an animal model for these syndromes. These studies are necessary to test and refine our model of the pathogenesis of these disorders. PND's provide examples of *bona fide* anti-breast tumor immunity in humans, and our hope is that the studies underway in this project will enable a detailed analysis of the mechanisms of successful anti-tumor immunity.

The methods of study in this project depend on the use of cloned genes (Sakai et al., 1990; Fathallah-Shaykh et al., 1991; Buckanovich et al., 1993) encoding the PND breast tumor antigens Nova and cdr2. The first specific aim is to assay clinical tumor specimens for the expression of PND genes and the presence of PND antigens. In some cases, it will also be appropriate to assay for co-factors that might be associated with the immune recognition of PND antigen (i.e. if we cannot establish a direct relationship between PND antigen expression and anti-tumor immunity). The second and third aims are complementary approaches aimed at establishing animal models for the breast tumor associated PND's, using breast tumor cell lines transfected with PND genes and transgenic mice making breast tumors expressing PND genes, respectively.

## BODY:

### ***Aim 1: Characterization of the Expression of PND Genes:***

A. PND expression in PND+ breast tumors. One test of the model presented for the pathogenesis of the PND's is that the expression of onconeural antigens is normally restricted to neurons, allowing them to be recognized by the immune system as "foreign" antigens when ectopically expressed in breast tumors. We have focused on the "Yo" antigen, which is expressed in breast and ovarian tumors. This antigen is normally expressed in Purkinje neurons and is targeted in patients with paraneoplastic cerebellar degeneration (PCD). Recently an additional neuronal gene termed cdr3, highly related to the cdr2 gene thought to encode the Yo tumor antigen, has been cloned (Posner et al., personal communication, unpublished data). To establish which cdr gene, if either, is expressed and thereby able to encode the 52kD Yo breast and ovarian tumor antigen, we obtained three clinical tumor specimens from patients with PCD. From one patient, we were also able to obtain a section of cerebellum (courtesy of Dr. J. Dalmau) to confirm that the patient had typical PCD. We performed immunohistochemical staining of a section of brain from patient 3 and a normal control using PCD antiserum. There is no cellular neuronal staining in the section of brain from the PCD, and all of the Purkinje neurons are absent (presumably destroyed), confirming that this patient suffered from typical immune-mediated PCD (Corradi et al., 1997). RNA was extracted from tumor samples from this and two other patients and the expression of cdr2 and cdr3 was evaluated by RT-PCR analysis. We found that each tumor expressed readily detectable cdr2 mRNA, but no detectable cdr3 mRNA (Corradi, et al., 1997). As a control, brain RNA assayed for expression of cdr2 and cdr3 mRNA was found to express readily detectable levels of both.

In addition to confirming that cdr2 is likely to be the relevant gene encoding the *bona fide* breast and ovarian "Yo" tumor antigen, we have pursued our studies of cdr2 mRNA expression. Surprisingly, we have found that cdr2 mRNA is abundant in all tissues, as assessed by Northern blot and RT-PCR analysis Corradi, et al., 1997. Moreover, sequencing of full length cDNA clones from brain and spleen revealed no changes in the sequence of the mRNA in either tissue. We also have shown that antibody to cdr2 only recognizes protein in brain and testis, as assessed by Western blot analysis (Figure 6B, Corradi, et al., 1997).

To confirm and extend these results, we have now completed immunohistochemical and *in situ* hybridization analysis of cdr2 protein and mRNA expression (Corradi, et al., 1997). These studies demonstrate that the cdr2 antigen is indeed expressed in a tissue-specific manner. Within the nervous system, both cdr2 protein and mRNA are found within discrete sets of neurons (primarily Purkinje cerebellar neurons, a few brainstem nuclei, and dorsal root ganglia). Outside of the nervous system cdr2 mRNA is widely expressed, although, surprisingly, with a high degree of tissue specificity within some tissues (e.g. splenic cortex, testicular spermatogoni). However, immunohistochemical analysis of Yo antigen expression revealed no expression outside of the nervous system except within the spermatogonia of the testis Corradi, et al., 1997. Notably, normal breast tissue or ovarian tissue did not express cdr2 antigen, while tumor tissue does (Darnell, 1996; Corradi, et al., 1997). We also note in this context that testis is normally the major immunologically isolated site outside of the nervous system.

Preliminary work indicated that the expression of the Nova antigen was normally restricted to neurons using PND antisera to assay protein expression (Buckanovich, et al., 1993). However, since we have found evidence for a second Nova gene family member, Nova-2 (Yang et al., 1998), we have pursued this observation at the level of expression of the Nova-1 gene, using a gene specific probe for *in situ* hybridization. These studies (Buckanovich et al., 1996) confirm that the Nova-1 gene and antigen are restricted to neurons at all points during the normal development of mice, and support the model for the pathogenesis of the PNDs outlined in the

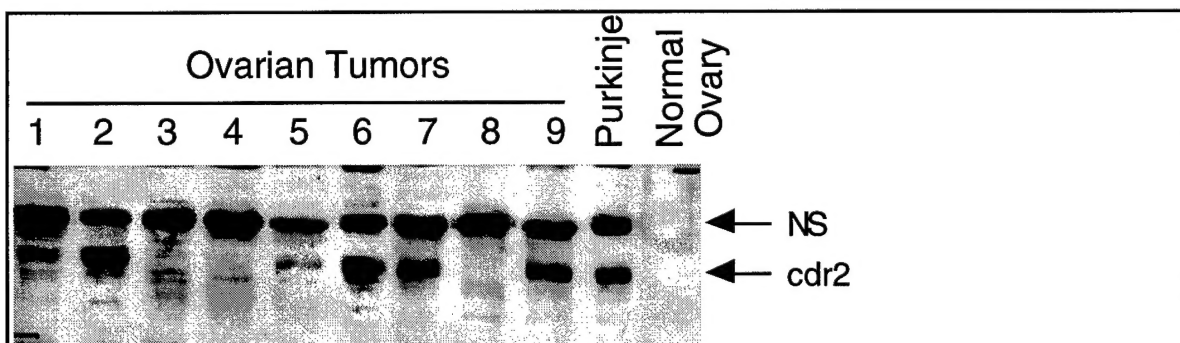
introduction. Expression of the Nova-2 gene also seems restricted to neurons, with the possible exception of an unidentified population of cells within the lung (Yang, et al., 1998).

To determine the specific expression pattern of Nova-2, and to determine which of the Nova genes encodes the paraneoplastic opsoclonus-myoclonus ataxia (POMA) breast tumor antigen, we have used the Nova-2 cDNA to predict a unique peptide fragment. Antibodies generated to this peptide have been made, and these discriminate completely between Nova-1 and Nova-2 fusion proteins. This antisera works to specifically recognize the neuronal Nova-2 antigen (Yang, et al., 1998). We found that Nova-2 is expressed in a pattern that is largely the reciprocal of Nova-1. Nova-2 is heavily expressed in neocortex and hippocampus, areas where there is no expression of Nova-1. These studies suggest that Nova-2 is the target neuronal antigen in breast cancer patients who develop dementia in association with antibodies to the Nova protein (Luque et al., 1991; Hormigo et al., 1994; Yang, et al., 1998).

#### PND expression in Non-PND tumors.

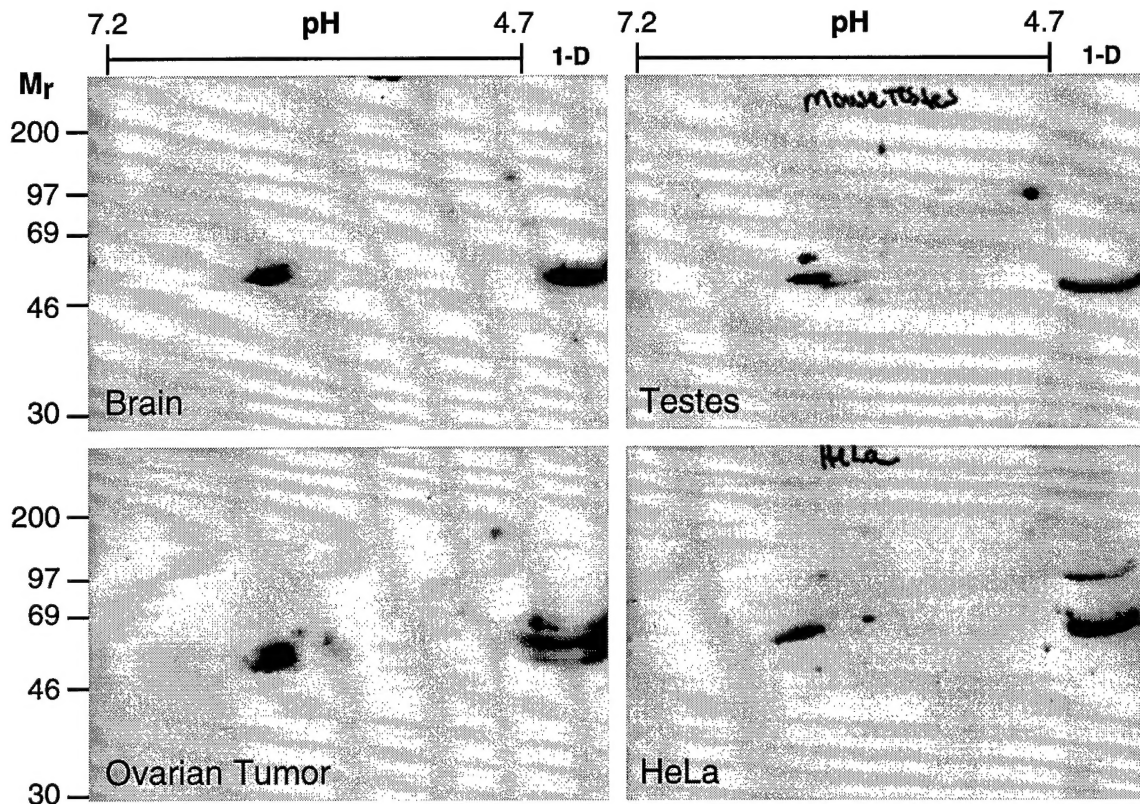
The observation that cdr2 antigen expression is tissue specific is consistent with the current model of PND pathogenesis, and is particularly significant for the current project. It indicates that the study of cdr2 mRNA in breast and ovarian tumor tissues, as originally proposed, is not the appropriate level of analysis. Rather, an analysis of cdr2 antigen expression, as assayed by Western blot and immunohistochemistry, will be the relevant focus of study for this antigen. We have now pursued this observation by examining expression of the cdr2 antigen from non-PND human tumor samples. We have found that ~60% of all human ovarian tumors appear to express the cdr2 antigen by Western blot analysis (N=16; Figure 1).

To determine whether the immunoreactive band co-migrating on one dimensional SDS-PAGE



**Figure 1. Western blot analysis of cdr2 expression in human ovarian tumors.** Protein extracts from 9 human ovarian tumors were run on Western blots and probed with biotinylated affinity purified PCD antibody. Strong cdr2 reactivity was evident in tumors 1, 2, 6, 7 and 9, as well as in extracts of human Purkinje neurons; there was no reactivity with a protein extract of normal human ovary. Reprobing the blot with an anti-tubulin antibody showed equal immunoreactive protein in each lane (including normal ovary; data not shown). A non-specific (NS) band was present in Purkinje extracts and ovarian tumors that reacted with streptavidin-peroxidase secondary alone (data not shown).

in tumor and brain is actually the same protein, we have also examined cdr2 expression by two-dimensional IEF/SDS-PAGE analysis. We find that brain, testis, and ovarian tumors and HeLa cells all express identically migrating PCD antigen reactive with affinity purified Yo antisera (Figure 2). These data indicate that the majority of human ovarian tumors express the cdr2 tumor antigen. We have also begun to look at human non-PND breast tumors, and find in a small sample that 25% of these tumors express cdr2 (N=4; data not shown).



**Figure 2. IEF/SDS-PAGE analysis of *cdr2* expression.** An immunoreactive band of identical  $M_r$  and  $pI$  is present in each indicated tissue. Extraneous cross reactive bands seen in standard SDS gels (1-D, as indicated) using HeLa or ovarian tumor extracts do not resolve on IEF. Identical results were obtained with human Purkinje cell extracts and affinity purified *cdr2* antibody (data not shown).

### ***Aim II-III. Animal Models of Breast Tumor Immunity***

**Problem #1 with Proposed Animal Model.** We proposed to establish a model for anti-breast tumor immunity in mice using a breast tumor cell line (RAC) transfected with the PND breast tumor antigens Nova and *cdr2*. For reasons that we do not understand, we have not been able to maintain stable RAC cell lines expressing *cdr2*. In several independent experiments, cells transfected with *cdr2* expression constructs and selectable markers (pcDNA3 or pMSG) were selected for 3-4 weeks in the presence of either G418 or gpt selection medium. In every case, drug resistant clones were obtained, but in no case did they express *cdr2* protein (assayed by Western blot analysis). This contrasts with our background data (presented in Figure 5, 1995 Report) demonstrating that these constructs do express high levels of *cdr2* in transient transfection assays. One way in which we are currently pursuing these observations by analyzing whether *cdr2* expression might cause functional changes in these cells that can affect cell growth. One such function would be impingement on cell cycle progression or viability in some tissue culture cells. In this regard, we have recently identified the c-Myc proto-oncogene as a protein binding partner for *cdr2* (Park et al., 1999). **These studies, discussed below, represent new studies performed in the past year.**

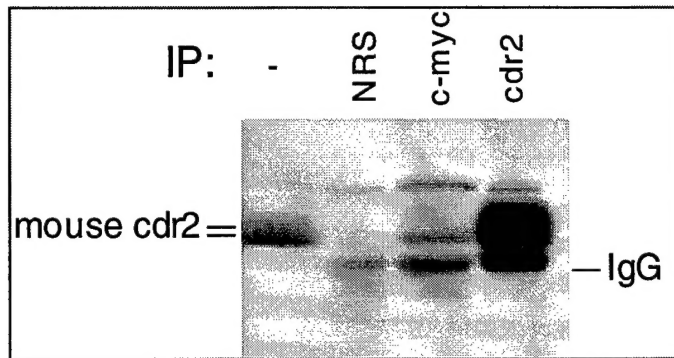
Unexpected identification of cdr2 interaction with c-Myc. The identification of c-Myc as a binding partner for cdr2 presented an unexpected direction for our work that is relevant to breast tumor biology. Myc acts as an oncogene in many tumor types, especially including the gynecologic tumors in which cdr2 is expressed. Our interest in the cdr2-Myc interaction was also heightened by our observations that cdr2 is the tumor antigen expressed in PCD tumors (Corradi et al., 1997) and that cdr2 is expressed in a wide range of gynecologic tumors (Figs 1-2).

We examined whether the biology of cdr2 might be relevant to breast and ovarian cancer. We were able to demonstrate that cdr2 and c-Myc proteins can be co-immunoprecipitated from mouse brain extracts (Fig 3), and that the two proteins colocalize in the cytoplasm of Purkinje neurons within the cerebellum (data not shown). We also demonstrated that cdr2 was able to antagonize the ability of c-Myc to induce transcription from an E-box containing promoter in heterologous cells (Fig 4).

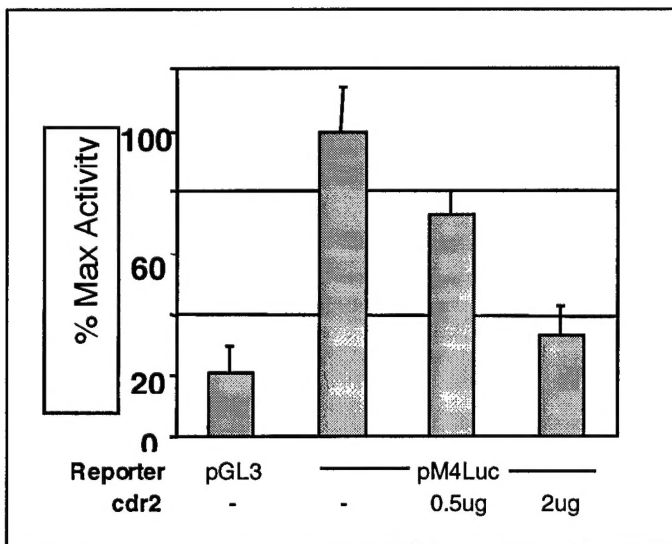
Since PCD antisera are known to recognize the cdr2 dimerization motif, we examined whether 6 different PCD antisera collected in the course of our studies (Aim 1) were able to affect the interaction between cdr2 and Myc. These data demonstrated that a characteristic of all 6 PCD antisera was that they are able to block the interaction between cdr2 and c-Myc (Figure 5).

We hypothesize that this might relate to the action of this antisera in the development of neuronal apoptosis and autoimmune neurologic disease. Conversely, if disruption of the interaction between cdr2 and Myc leads to neuronal apoptosis, the normal action of cdr2 may be to prevent Myc-dependent apoptotic pathways (Evan and Littlewood, 1998). These studies thus open new questions and testable hypotheses regarding the role of cdr2 in regulating Myc oncogene function in breast and ovarian cancer.

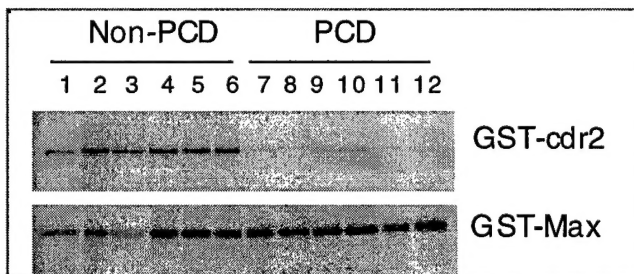




**Figure 3.** cdr2 co-immunoprecipitates with c-myc in mouse cerebellum. Mouse cerebellar homogenate was precipitated with the indicated antibodies. Immune complexes were analyzed by Western blot using PCD patient serum. Lane 1 is a Western blot of the cerebellar lysate.



**Figure 4.** cdr2 represses Myc transcriptional activity. NIH3T3 cells were transiently transfected with pGL3basic (Promega) or pM4Luciferase (pM4luc) reporter plasmids. pcDNAJyc and the indicated amount of pcDNA3-cdr2 plasmid. Co-transfected cdr2 inhibited Myc-dependent luciferase activity in a titratable manner. The results shown represent the average of triplicate transfections.

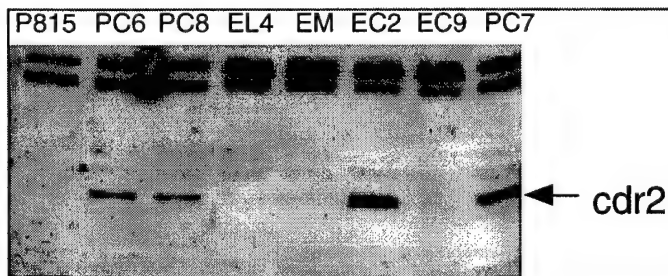


**Figure 5. Inhibition of cdr2:Myc interaction by PCD patients' sera.** GST-cdr2 or GST-Max fusion proteins in solution were immobilized on glutathione-sepharose beads, preincubated with sera from six different PCD patients or non-PCD controls, washed, and then mixed with  $^{35}$ S-methionine labeled Myc protein. Specifically bound Myc protein was assessed by SDS-PAGE and fluorography. Quantitation of the ratio of cdr2 to Max protein precipitated in each sample indicated that PCD sera inhibited  $^{35}$ S-Myc pull downs by an average of 5.5 (range 4.9-7.1) fold.

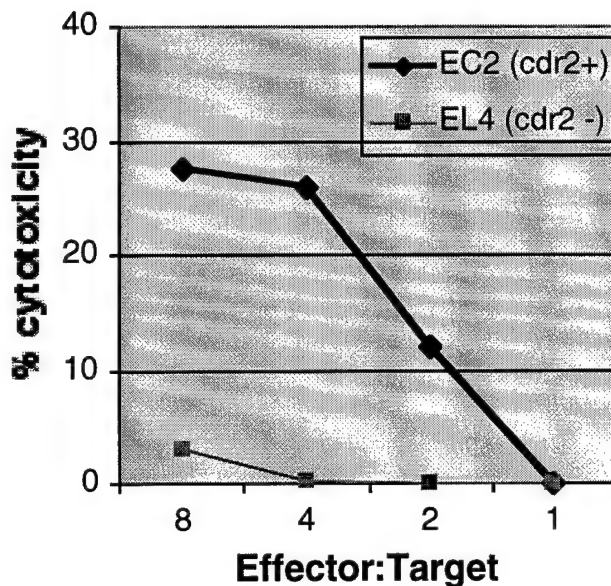
**Problem #2 with Proposed Animal Model.** In addition to the difficulties transfecting RAC cells, we also proposed using currently available breast tumor transgenic mice (MMTV-wnt, neu and myc) to test the role of PND antigens in anti-tumor immunity. To adequately control these studies, we planned to adoptively transfer transgenic breast tumors that either do or do not express PND antigens into naive syngeneic mice. The background work to establish such experiments includes an analysis of transgenic breast tumors for endogenous PND antigen expression, followed by construction of MMTV-driven PND expression vectors. To date, we have obtained MMTV-myc and MMTV-wnt breast tumor tissue (from Charles River and H. Varmus, respectively) for analysis of PND expression. We have analyzed these tumors for Nova antigen expression, and find no evidence that they express this antigen (data not shown). Initial studies indicated that each tumor type expressed *cdr2* mRNA (data not shown); however, given our results indicating that *cdr2* is under tight translational control, we also evaluated *cdr2* antigen expression in these tumors by Western blot analysis. We found that the myc, and possibly wnt tumors, appear to express *cdr2* antigen by one-dimensional SDS-PAGE analysis. While this may be consistent with our observation that up to 60% of non-PND tumors express *cdr2*, it would also complicate our animal model studies.

**New approaches to animal models of PND tumor immunity.** A new approach to overcome the problem with RAC cells and the proposed transgenic breast tumors has been to utilize is to use EL4 (B-cell lymphoma) cells and P815 (SVB6 transformed) cells, which are mouse cell lines of H-2D<sup>b</sup> and H-2D<sup>d</sup> haplotype, respectively, to generate animal models of PND. These studies, **which represent new work performed over the past year**, have established a new approach to our animal model that seems very promising. We now succeeded in generating stably transformed tumor cell lines that express *cdr2*, and can use these in comparative experiments with parental cell lines that do not express any detectable *cdr2* (see Figure 6). Moreover, we have demonstrated that inoculation of mice with these cells is able to generate *cdr2*-specific CTLs. Given our human experiments (see below), these studies suggest that we are now beginning to generate an animal model that closely parallels the human PND.

Studies in the immunology laboratory of our colleagues Drs. Ralph Steinman and Nina Bhardwaj at the Rockefeller University have recently indicated that cellular apoptosis may lead to the effective presentation of intracellular antigen to dendritic cells (Albert et al., 1998b). We now plan to pursue this observation in our animal models by taking stable *cdr2* expressing cell lines, apoptosing them (by UV irradiation), and using them as adjuvants to stimulate T cell immunity in animals. We will then assay for anti-tumor immunity by implanting tumor producing *cdr2*-expressing cell lines at distant sites as planned. **In preliminary experiments performed over the past year**, we have now demonstrated that apoptotic EC2 cells are able to generate *cdr2*-specific CTLs in this inoculation protocol (Figure 7). These studies almost exactly parallel the originally proposed experiments for generating an animal model for PCD, but do so using the information gained from human clinical studies and from our unsuccessful attempts to use RAC cells to generate models of breast tumor immunity.



**Figure 6.** Western blot analysis of *cdr2* expression in stably transfected P815 cell clones (PC6-8) or EL4 cell clones (EM, EC2, EC9). Cell clones are EC2 and PC7 express relatively high levels of *cdr2*.



**Figure 7.** Innoculation of EC2 tumor cells stimulates cdr2-specific tumor immunity in mice. C57Bl/6 mice (H-2<sup>b</sup>) were inoculated with irradiated cdr2-expressing PC7 tumor cells (H-2<sup>d</sup>). Splenic T cells were stimulated in vivo with EC2 cells, and assayed in <sup>51</sup>Cr cytotoxicity assays against EL4 (cdr2<sup>-</sup>) or EC2 (cdr2<sup>+</sup>) target cells.

**Problem #3 with Proposed Animal Model.** One of the challenges in the original plans to generate an animal model of breast tumor immunity presented in the original proposal was that we did not know, a priori, that cellular immunity played a role in the pathogenesis of these disorders. We have now demonstrated that cdr2-specific CTLs play a major role in the pathogenesis of PCD by examining human peripheral blood mononuclear cells obtained from PCD patients. Although the human studies were not performed as part of this proposal, a necessary prerequisite to these experiments was our data, accomplished in Aim I, establishing cdr2 as the breast and ovarian tumor antigen expressed in PCD patients. Moreover, the Western blot assays used to definitively diagnose the presence of the cdr2 antibody (Albert et al., 1998a; Figure 1) were necessary in these studies to determine that patients with a clinical diagnosis of PCD did in fact harbor the cdr2 antibody. For these reasons, these studies, **which were published in the last year (Albert, et al., 1998a; included in Addendum)**, acknowledged support from the DOD grant. The significance of these studies for the development of an animal model for PCD are discussed below.

Our studies of human PCD patients revealed that cdr2-specific T cells capable of killing cdr2-expressing targets are present in patients with PCD and naturally occurring tumor immunity. All PCD tumors examined to date, including one in this study, express the cdr2 antigen. Thus the cells we have identified are of great interest to the understanding of mechanisms by which immunity to breast cancer may operate in humans. We found, for example, that these cells appear to be classic  $\alpha/\beta$  TCR, recognizing targets in an MHC class I restricted manner. Moreover, we demonstrated very efficient killing of targets following the challenge of autologous dendritic cells (DCs) with apoptotic tumor cells expressing the cdr2 antigen (HeLa cells; Figure 3 in Albert, et al., 1998a).

These observations indicate that we are on the right course in attempting to develop an animal model of PCD-associated breast tumor immunity in the experiments outlined above. Specifically, we are encouraged that our EL4 and P815 transfected cell experiments have been able to generate cdr2-specific CTLs in mice. Moreover, our human data suggest that induction of cdr2 recall immunity by immunization with apoptotic tumor cells may be a particularly effective and important means of inducing tumor immunity. These studies will contribute significantly to the completion of the Aims of this proposal.



## CONCLUSIONS:

The completed research demonstrates that the Nova and cdr2 PND antigens are both normally expressed in immune-privileged sites (Buckanovich et al., 1993; Buckanovich et al., 1996; Corradi, et al., 1997; Yang, et al., 1998). This supports our general model for the pathogenesis of the breast tumor immunity seen in PND patients (Darnell, 1996). That is, the immune system appears to recognize these antigens in the context of breast tumor cells as foreign antigens; their normal restriction to neurons (and testis for cdr2) sequesters them from immune surveillance and prevents their recognition as self antigens. Strikingly, we have found that the mechanisms for sequestering Nova and cdr2 antigens to neurons are very different. Nova mRNA and protein expression are found to be restricted to neurons at all developmental times. In contrast, cdr2 mRNA is widely expressed in various tissues; within the nervous system its expression is restricted to Purkinje cerebellar neurons, brainstem neurons and dorsal root ganglia, but outside the nervous system many tissues express the gene. Tight restriction of cdr2 antigen expression to neurons and testis is demonstrated by Western blot and immunohistochemical analysis, leading to the conclusion that post-transcriptional (likely translational) regulatory mechanisms restrict the expression of cdr2 to immune-privileged sites.

These studies suggest that the cdr2 gene is under strict regulatory control. Our findings are reminiscent of observations of neuron-specific gene transcription mediated by transcriptional silencers outside of the nervous system (Chong et al., 1995). Moreover, a direct precedent for our findings can be found in studies of the expression of the BTEB transcription factor. BTEB mRNA is made in many tissues but, due to 5'-UTR translational control elements, the protein is made only in brain and testis (Imataka et al., 1994).

Our observations have important implications for the role of cdr2 in breast and ovarian tumor biology, suggesting for example that tumors that ectopically express cdr2 antigen have dysregulated translational control mechanisms. Such dysregulation has not been previously observed in breast tumors, and suggests a possible target for therapeutic intervention. Moreover, these observations suggest a means for identifying this control mechanism. We have begun a systematic analysis of cdr2 translational control, work that should bear on the mechanism of its induction in breast tumors.

By substantiating the observation that the cdr2 antigen is expressed *de novo* in a significant number of ovarian and perhaps breast tumors, we have accomplished several of the goals of this work. First, we have established the identification of these antigens as bona fide tumor markers. Second, by expanding their utility from markers in rare patients with PND to markers in many breast and ovarian tumor patients, we will have greatly broadened the practical significance of this observation.

Our studies of cdr2 tumor expression have heightened our interest in the biologic function cdr2 may play in gynecologic tumors. These studies led to the identification of c-Myc as a binding partner for cdr2. Studies using PCD patient antisera suggest that these antisera abrogate the action of cdr2 to inhibit c-Myc transcription. These studies have led to a model in which cdr2 may normally act to block Myc pro-apoptotic actions in neurons, and lead to testable hypotheses regarding the function of the interactions between cdr2 and Myc in gynecologic tumors.

Finally, we are now interested in exploring the significance of our results to anti-tumor immunity. Specifically, we have demonstrated the presence of cdr2-specific CTLs in PCD patients with naturally occurring breast tumor immunity (Albert, et al., 1998a). Moreover, we have found from the studies in this work that large numbers of breast and ovarian cancer from neurologically normal individuals express the cdr2 antigen, and most (or perhaps all) small cell lung cancers (SCLCa) express the Hu onconeural antigen. Up to 15% of these neurologically normal SCLCa patients harbor low titer antibodies to the Hu antigen. Remarkably, this subset of SCLCa patients also have limited disease (Dalmau et al., 1990), as well as an associated

increased complete response to therapy and improved survival (Graus et al., in press). By analogy, then, our observation that large numbers of ovarian and perhaps breast tumor patients express the cdr2 and perhaps Nova onconeural antigens predicts a simple way to identify patients with inherent anti-tumor immunity without neurologic disease. We hypothesize that some of the patients (perhaps 15%) whose tumors express these onconeural antigens will have evidence of active anti-tumor immunity--limited disease, complete response to therapy, and/or improved survival. To establish support for this hypothesis, however, we will need to generate a sensitive assay for anti-cdr2 or anti-Nova low level immunity. This would be established using the recombinant onconeural fusion proteins. One complication that would need to be addressed in setting up such a study is the insolubility of the cdr2 fusion protein. One straightforward solution to this problem would be to express the protein in baculovirus, a project that has been recently accomplished in the laboratory. In addition, we will need to establish CTL assays for these antigens, which will be based on our successful establishment of assays for cdr2-specific CTLs (Albert, et al., 1998a).

## METHODS

### *Tumor RNA Extraction and RT-PCR.*

Frozen samples of ovarian tumors removed from patients with Yo-positive PCD were obtained from the Memorial Sloan Kettering Cancer Center. 100mg sections were used for purification of either total or poly A+ RNA. Total RNA was prepared by the method of Chomcynski and Sacchi (Chomcynski and Sacchi, 1987), and the samples were subsequently treated with RQ1 RNase-free DNase (Promega, Madison, WI) before RT-PCR analysis. For the purification of poly A+ RNA, the section was homogenized by sonication in 400µl extraction buffer (4M guanidinium thiocyanate, 0.1M Tris-HCl pH 8.0, 1% dithiothreitol, 0.5% lauryl sarcosinate), 800µl of binding buffer (0.1M Tris-HCl pH 8.0, 0.4M LiCl, 20mM EDTA) was added and the lysate was centrifuged at 18,000 x g for five minutes. Magnetic Dynabeads Oligo (dT)<sub>25</sub> (DynaL, Inc., Great Neck, NY) were prepared by washing 300µl of beads once with 200µl Binding Buffer. The lysate supernatant was added to the Dynabeads, mixed, and incubated at room temperature for 3-5 minutes. A magnet was used to immobilize the beads, the supernatant was removed and the beads were washed three times with 0.5 ml wash buffer (10mM Tris-HCl pH 8.0, 0.15M LiCl, 1mM EDTA). PolyA+ RNA was then eluted in 20µl 2mM EDTA pH 8.0 at 65°C for two minutes.

For RT-PCR reactions 2µl tumor polyA+ RNA, 1-2µg total tumor RNA or 20ng polyA+ RNA from normal tissues (CLONTECH Laboratories, Inc., Palo Alto, CA) were denatured at 70°C for ten minutes and placed on ice. The RNA was reverse transcribed using random hexanucleotide primers (Boehringer Mannheim, Indianapolis, IN) and Superscript reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42°C for 50 minutes and the reaction stopped by incubation at 95°C for 5 minutes. One-tenth of the first strand cDNA sample was used as template for a PCR reaction using AmpliTaq polymerase (Perkin Elmer Corp., Norfolk, CT) and the following forward and reverse oligonucleotide primers corresponding to cdr2: 5'-TGAATGGAGTTGAGA AGCTGGTG-3' and 5'-GAGATGCCCCCTCTGTTTCAC AG-3'; and cdr3: 5'-CATTGAGCGCCTCCAGGCT-3' and 5'-AGCTCCTTGAGG CAGGGGAA-3'. The product was amplified for 35 cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute). PCR products were labeled by addition of trace amounts of  $\alpha^{32}\text{P}$ -dCTP (Amersham Life Science Inc., Arlington Heights, IL) to the reaction mixtures and were loaded on a 10% non-denaturing acrylamide gel and visualized by autoradiography.

### *Two-dimensional gel electrophoresis.*

Cerebella and testes from Sm/Ckc mice were homogenized in 2D lysis buffer (9.5M urea, 2% NP-40, 5%  $\beta$ -mercaptoethanol, 2% Biolyte ampholytes (BioRad Labs, Hercules, CA) consisting of 75% 3/5 range and 25% 3/10 range Biolytes). The lysate was clarified by centrifugation at 2100 x g for five minutes, and protein concentrations were adjusted with 2D lysis buffer. Isoelectric focusing (IEF) gels were performed essentially by the method of O'Farrell (O'Farrell, 1975). 0.75mm IEF slab gels (9.2M urea, 4% acrylamide (ReadySol IEF, Pharmacia Biotech), 2% NP-40, and 5% Biolyte ampholytes) were loaded with 40µg of total protein per lane, and the samples were covered with sample overlay buffer (7% urea, 2.5% ampholytes, 5%  $\beta$ -mercaptoethanol). The gels were run using 0.01M H<sub>3</sub>PO<sub>4</sub> and 0.02M NaOH buffers as described (O'Farrell, 1975) at 4W constant power. Voltage was limited to 700V and gels were run for 1800V-hr. Lanes containing the samples were cut from the gel, equilibrated with 1X SDS sample buffer for five minutes, and loaded horizontally onto a 1mM 9% SDS-PAGE gel with a 3% stacking gel. A single well was loaded with 40µg protein extract in SDS sample buffer for one-dimensional analysis. Gels were transferred to nitrocellulose and probed with PCD antisera.

### ***Immunoprecipitation and western blot***

Mouse cerebellum was homogenized in LS lysis buffer (20mM HEPES, pH7.5, 100mM KCl, 10mM MgCl<sub>2</sub>, 5mM dithiothreitol (DTT), 0.2% NP-40, 1, 2μg/ml aprotinin, 0.2mM phenylmethylsulfonyl fluoride (PMSF)) using a dounce homogenizer. Homogenates were sonicated briefly and soluble fractions were collected after spinning. The lysates were precleared with Protein A-sepharose and normal rabbit sera, and then precipitated with PCD CSF or anti-Myc rabbit polyclonal antibody (Upstate Biotechnology). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Blotted proteins were analyzed using anti-cdr2 patients' sera or anti-Myc mouse monoclonal antibody (C-33, Santa Cruz Biotechnology). Each protein was visualized in the blot using HRP-conjugated anti-human or anti-mouse IgG and ECL chemiluminescence kit (Amersham).

### ***Transcription Assay (CAT assay and luciferase assay)***

The pSpMyc and (+/-)M4minCAT plasmids used in transfection assays were kindly provided by Dr. R. Eisenman. For CAT assays, transfected cells were lysed in 0.25M Tris HCl, pH7.5 by repeated freezing and thawing. Cytoplasmic extracts were mixed with CAT assay buffer (2μCi/ml <sup>14</sup>C-chloramphenicol, 0.25mg/ml n-butyryl CoA, 16.6mM Tris HCl, pH8.0), and incubated at 37°C. To isolate the acetylated <sup>14</sup>C-chloramphenicol, samples were extracted by 2:1 mixture of tetramethyl pentadecane (TMPD)/xylene. Radioactivity in the extracted organic phase was measured using a liquid scintillation counter.

Luciferase assays were done using a luciferase assay kit (Promega) as described by the manufacturer. Transfection efficiency was normalized by measuring the β-gal activity derived from cotransfection with a CMV-lacZ reporter construct, and, in some instances normalizing the number of cells transfected using a pEGFP reporter (Clontech). To measure the β-gal activity, cytoplasmic extracts were mixed with Buffer A (100mM NaH<sub>2</sub>PO<sub>4</sub>, pH7.5, 10mM KCl, 1mM MgSO<sub>4</sub>, 50mM β-mercaptoethanol) and 4mg/ml O-nitrophenyl D-β-galactopyranoside (ONPG). After incubation, reactions were stopped by adding 1M Na<sub>2</sub>CO<sub>3</sub> and the absorbance at 420nm was measured.

### ***Competition Assay***

GST-cdr2 or GST-Max fusion proteins in solution were immobilized on glutathione-sepharose beads and incubated with patients' sera. After washing, *in vitro* translated <sup>35</sup>S-Myc proteins were added to each tube. After washing again, proteins present on GST-cdr2 or GST-Max sepharose beads were analyzed by SDS-PAGE and fluorography.

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## Appendix

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# **Tumor-specific killer cells in paraneoplastic cerebellar degeneration**

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# Tumor-specific killer cells in paraneoplastic cerebellar degeneration

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Models for immune-mediated tumor regression in mice have defined an essential role for cytotoxic T lymphocytes (CTLs); however, naturally occurring tumor immunity in humans is poorly understood<sup>1</sup>. Patients with paraneoplastic cerebellar degeneration (PCD) provide an opportunity to explore the mechanisms underlying tumor immunity to breast and ovarian cancer. Although tumor immunity and autoimmune neuronal degeneration in PCD correlates with a specific antibody response to the tumor and brain antigen cdr2<sup>2,3</sup>, this humoral response has not been shown to be pathogenic<sup>3,4</sup>. Here we present evidence for a specific cellular immune response in PCD patients. We have detected expanded populations of MHC class I-restricted cdr2-specific CTLs in the blood of 3/3 HLA-A2.1<sup>+</sup> PCD patients, providing the first description, to our knowledge, of tumor-specific CTLs using primary human cells in a simple recall assay. Cross-presentation of apoptotic cells by dendritic cells also led to a potent CTL response. These results indicate a model whereby immature dendritic cells that engulf apoptotic tumor cells can mature and migrate to draining lymph organs where they could induce a CTL response to tissue-restricted antigens. In PCD, peripheral activation of cdr2-specific CTLs is likely to contribute to the subsequent development of the autoimmune neuronal degeneration.

We examined the nature of the immune responses of three HLA-A2.1<sup>+</sup> PCD patients (patients 1–3) and one HLA-A2.1<sup>−</sup> PCD patient (patient 4). Patient 1 was seen during the acute phase of the disorder, and patients 2–4 had chronic disease at admission; they were seen 18 days, 9 months, 6 months and 5 months, respectively, after the onset of cerebellar dysfunction. After being diagnosed with PCD, all four patients were found to have gynecologic cancers: patient 1 had breast cancer and patients 2–4 had ovarian carcinoma. PCD is characterized by the presence of a high-titer antibody that is present in a patient's serum and spinal fluid; this antibody recognizes target antigens in Purkinje neurons and in gynecologic tumors (onconeural antigens; ref. 3). PCD antiserum has been used to clone cDNAs encoding immunoreactive antigens cdr1–3 (refs. 5–7), but PCD tumors express only the cdr2 antigen<sup>8</sup>. Thus, we confirmed the diagnosis of PCD in these patients by demonstrating the presence of high-titer cdr2 antibodies reactive with cloned cdr2 fusion protein (Fig. 1 and data not shown). For patient 1, a tumor block was available; by western blot analysis, the tumor was shown to express cdr2 (data not shown). Historically, all PCD tumors analyzed have been shown to express the cdr2 antigen<sup>8,9</sup>. Expression of cdr2 is normally tightly restricted to immune-privileged sites (neurons and testis)(ref. 8).

Initially, the discovery of onconeural antibodies led to the pro-

posal that PCD is an autoimmune disorder mediated by the humoral arm of the immune system. However, several observations indicate that cdr2 antibodies are not sufficient to cause disease. Treatments that reduce antibody titers are ineffective<sup>4</sup> and passive transfer of antibody does not reproduce the disorder in animals<sup>3</sup>. Furthermore, cdr2 is an intracellular protein, and it is unclear how such an antibody could mediate disruption of cellular function. To determine whether CD8<sup>+</sup> CTLs are involved in tumor immunity in PCD, we analyzed lymphocytes from the peripheral blood of patients. Peptide epitopes derived from cdr2 were 'pulsed' onto target cells, and antigen-specific cytotoxicity was measured in a standard <sup>51</sup>Cr-release assay. In patient 1, cdr2-specific CTLs were detected with specificity for the cdr2-2 peptide, and, to a lesser extent, the cdr2-1 peptide (Fig. 2a). This response was titratable and specific for acute PCD, as no response was detected in an HLA-A2.1<sup>+</sup> normal control (Fig. 2a) or in the patients with chronic PCD (data not shown).

To determine whether memory T cells were present in the peripheral blood of PCD patients, we established an *in vitro* recall assay. We prepared mature terminally differentiated dendritic cells<sup>10</sup> (DCs). The DCs generated had a typical stellate morphology, were nonadherent, expressed characteristic maturation markers (CD83), and had potent T cell-stimulating capacity in mixed leukocyte reactions at stimulator-to-responder ratios of 300:1 or less (data not shown). These blood-derived DCs were 'pulsed' with four different cdr2 peptides and co-cultured with purified syngeneic T cells. After 7 days, responding T cells were tested for cytolytic activity specific for cdr2 epitopes using 'peptide-pulsed' T2 (TAP<sup>−/−</sup>, HLA-A2.1<sup>+</sup>, class II<sup>−</sup>) cells as targets. In patients 2 and 3 (with chronic PCD), cdr2-specific CTLs were detected (Fig. 2b) using the cdr2-1 and cdr2-2 peptides. This CTL activity was not detected in the patient with acute PCD (patient 1), the HLA-A2.1<sup>−</sup> chronic PCD patient (patient 4), nor in five HLA-A2.1<sup>+</sup> control individuals tested (Fig. 2b and data not shown). As a positive control for these experiments, CTL responses specific for the immunodominant HLA-A2.1 epitope derived from the influenza matrix protein were determined (data not shown). Taken together, these data demonstrate the presence of an expanded population of class I-restricted cdr2-specific CTLs in the blood of both acute and chronically ill PCD patients.

To better understand how a tumor-specific antigen such as cdr2 might activate naive CTLs, we used an *in vitro* model for cross-presentation<sup>11</sup>. Apoptotic cells have been shown to serve as an essential trigger for the cross-presentation of epitopes derived from tissue-restricted antigens onto class I MHC molecules of professional antigen-presenting cells. HeLa cells, which express cdr2 (ref. 6), served as a source of antigen, and DCs were used as

**Fig. 1** Western blot analysis of serum and CSF immunoreactivity to cdr2. Sera and CSF of patient 1 (lanes 1 and 4) and patient 2 (lanes 2 and 5) were tested for immunoreactivity to the cloned cdr2 fusion protein. As a specificity control, serum from a patient with an irrelevant PND (Hu syndrome) was also blotted (lane 3). Serum and CSF from patient 3 showed similar immunoreactivity (data not shown).



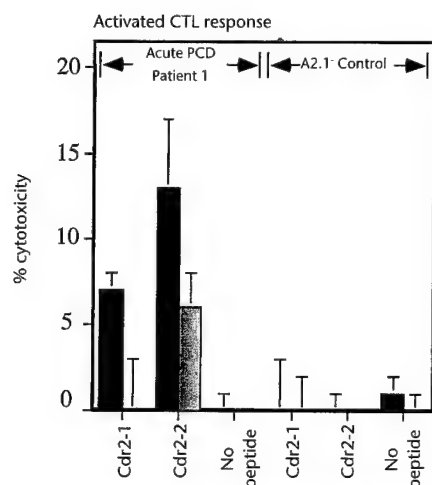
antigen-presenting cells for the induction of CTLs from the peripheral blood of patients with PCD. HeLa cells were induced to undergo apoptosis using ultraviolet B irradiation<sup>11</sup>. After 6–8 hours, DCs and T cells purified from patients 1 and 4 were added to the HeLa cell cultures. Responding T cells were collected after 7 days, and cytolytic activity specific for cdr2 epitopes was tested (Fig. 3). CTL activity specific for cdr2 epitopes was found in the HLA-A2.1<sup>+</sup> PCD patient (patient 1), but not in the HLA-A2.1<sup>-</sup> individual (patient 4), indicating that cell killing was MHC class I-restricted. CTLs were not generated to a control peptide (influenza matrix peptide, MP), indicating antigen specificity of the response. Reactivity was not detected against cdr2-2 peptide in this assay (data not shown), for reasons not yet understood. The percent killing demonstrated here is substantially higher than that detected using 'peptide-pulsed' DCs. We believe that the potent CTL response reflects increased efficiency by which antigens derived from apoptotic cells charge MHC-I on DCs relative to exogenous peptide (K. Inaba *et al.*, submitted). Additionally, the ability to stimulate CD4<sup>+</sup> T cells in these co-cultures may contribute to enhanced induction of cdr2-specific CTLs (ref. 12). Although a peptide derived from a HeLa antigen

other than cdr2 possibly served as the epitope for the activation of the cdr2-specific T cells, the use of 'unpulsed' and 'peptide-pulsed' T2 cells as targets in the <sup>51</sup>Cr-release assay conclusively demonstrates the expansion of HLA-A2.1-restricted cdr2-specific cells in patient 1.

It is well-documented through animal models and clinical experience that the immune system can recognize and kill tumor cells<sup>1</sup>. CTLs are believed to have an essential role in this immune response, and the induction of tumor antigen-specific CTLs for immunotherapy constitutes an emerging strategy for treating cancer patients. However, expanded populations of tumor-specific CTLs have not previously been found in humans. In melanoma, CTL precursors specific for tumor antigens are not expanded in patients with cancer compared with those in normal individuals<sup>13</sup>. Indeed, established T-cell lines specific from defined tumor antigens could only be generated by repeated stimulation with antigen, reflecting *in vitro* priming rather than a true recall response<sup>1</sup>. This is in contrast to individuals who are infected with a virus such as HIV or influenza, in whom CTL precursor numbers are greatly expanded<sup>14–16</sup>. One reason for this discrepancy may be that the cancer patients studied have not mounted effective immune responses to their tumors; tumors such as melanoma contain restricted antigens for T cells, but they fail to trigger immunity.

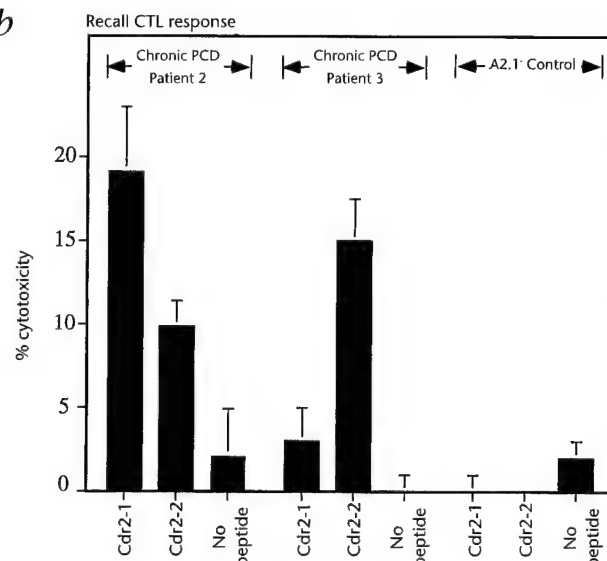
Here we have examined the blood of PCD patients for evidence of cellular immunity, providing the first evidence, to our knowledge, for tumor-specific CTLs in patients with clinically evident tumor immunity. Paraneoplastic neurologic disorders (PNDs) are associated with tumor cell expression of neuron-specific proteins, and cDNAs encoding the respective onconeural antigens have been cloned<sup>3</sup>. PND patients typically come to clinical attention with severe neurologic dysfunction,

a

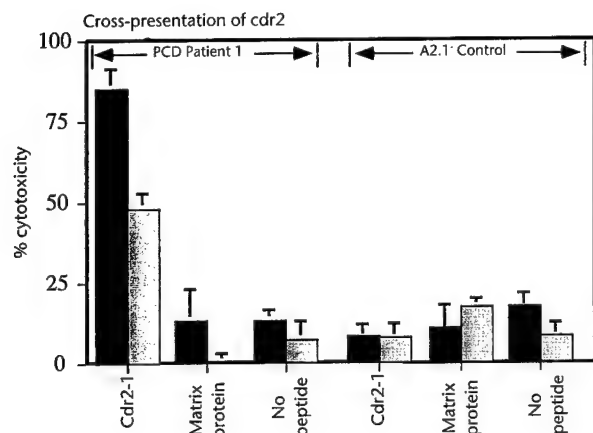


**Fig. 2** Peripheral blood of PCD patients contains cdr2-specific killer cells. T cells were isolated from the peripheral blood of acute (a) and chronic (b) HLA-A2.1<sup>+</sup> PCD patients. **a**, These purified T cells were used directly in a <sup>51</sup>Cr-release assay using 'peptide-pulsed' T2 cells as targets. Peptides cdr2-1 and cdr2-2 were predicted based on known anchor residues for the HLA-A2.1 binding groove. Effector:Target ratios are 100:1 (filled bars) and 30:1 (shaded bar). **b**, Blood-derived dendritic cells were generated from PCD and HLA-A2.1<sup>+</sup> matched control individuals. These DCs were 'pulsed' with the cdr2-1 and cdr2-2 peptides and co-cultured

b



with T cells. After 7 days, the responding T cells were tested for cytolytic activity specific for cdr2 as determined in a standard <sup>51</sup>Cr-release assay. The HLA-A2.1 immunodominant epitope derived from the influenza matrix protein served as a positive control for the generation of a CTL recall response (data not shown). Effector:Target ratio is 20:1. In **a** and **b**, percent cytotoxicity is measured as a function of spontaneous and total release. Background killing of target cells was 0–2% in all groups. Results are representative of four experiments, and each value represents the mean from triplicate wells.



**Fig. 3** Enhanced CTL activity is detected when apoptotic HeLa cells are the source of cdr2 epitopes. HeLa cells (filled bar),  $1 \times 10^4$ ; shaded bar,  $1 \times 10^3$  were ultraviolet B-irradiated to induce apoptosis, and DCs and T cells purified from a PCD patient were then added the apoptotic HeLa cells. Responding T cells were tested in a cytotoxicity assay. Percent killing of 'peptide-pulsed' T2 cells is shown using both no peptide and MP as negative controls. Patient 4, who is HLA-A2.1<sup>+</sup>, had no detectable cdr2-specific response. Data are representative of two experiments, and each value represents the mean from triplicate wells.

unaware that they have a tumor, and in some cases PND-associated tumors have been documented to regress with the onset of autoimmune neurologic disease<sup>17</sup>. Among patients with PCD, two-thirds present with neurologic symptoms before the diagnosis of cancer, and nearly 90% have limited oncologic disease when diagnosed; by comparison, only 50–60% of unselected breast cancer patients and 25% of ovarian cancer patients present with limited stage disease<sup>18</sup>.

In three of three HLA-A2.1<sup>+</sup> PCD patients, we have found cytotoxic T cells that specifically lyse target cells presenting HLA-A2.1<sup>+</sup>-restricted peptides derived from the PCD cdr2 antigen. Given that tumor cells themselves are unable to activate naive CTLs (ref. 19), professional antigen-presenting cells are likely to be essential for the induction of cdr2-specific CTLs in PCD (ref. 20). Based on our *in vitro* data (Fig. 3) and the *in vivo* models of others<sup>19</sup>, cross-presentation of tumor antigens could account for the initial stimulation of the CTLs in PCD. DCs can acquire apoptotic cells and effectively cross-present intracellular viral antigens derived from the apoptotic material on MHC I, stimulating class I-restricted CTLs (ref. 11). We have extended these observations by using cdr2-expressing apoptotic tumor cells as a source of antigenic material.

These observations indicate a mechanism whereby tumor antigen-specific T cells may be generated after expression of restricted antigens. Apoptotic tumor cells phagocytosed by peripheral tissue dendritic cells may cross-present epitopes derived from such tumor antigens on class I MHC molecules. After migrating to a draining lymph node, such DCs might engage antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. After being activated, such T cells may return to the tumor site and lyse tumor cells. In PCD, cdr2 may be an effective antigen for the induction of tumor immunity, due in part to the absolute restriction of its expression to immune-privileged sites throughout development<sup>8</sup>. Additional factors are also necessary for the immune response, including tumor cell expression of MHC-I (found in a high percentage of PND-associated tumors<sup>21</sup>) and lack of tumor-cell ex-

pression of proteins capable of inducing T-cell tolerance, such as Fas ligand<sup>22</sup>.

The detection of cdr2-specific CTLs in PCD patients indicates a new model for PCD pathogenesis. We suggest that cdr2-specific T cells are induced by the tumor cell expression of a protein normally expressed in immune-privileged tissues, leading to the observed tumor immunity. Such cells are likely to contribute to neuronal degeneration in PCD; this is supported by reports of MHC I expression in neurons<sup>23</sup>, including Purkinje neurons, and by detection of CTLs in the CSF of PCD patients early in the course of disease (M.L.A. and R.B.D., unpublished data).

## Methods

**Monoclonal antibodies.** Monoclonal antibodies to these antigens were used: CD8, CD14, HLA-DR (Becton Dickinson, San Jose, California) and CD83 (Coulter, Hialeah, Florida).

**Clinical samples.** All patients were self-referred after a diagnosis of PCD, and were seen at The Rockefeller University Hospital General Clinical Research Center. After informed consent was obtained, blood was drawn for analysis. Patient 1 was seen 18 days after the onset of cerebellar symptoms. At that time, she was able to speak with considerable dysarthria, could use her hands to pick up large objects, but otherwise she was completely bedridden with pancerebellar signs and symptoms. Patients 2–4 had chronic cerebellar disease and were clinically typical of individuals with PCD.

**Generation of mononuclear cell subsets.** Blood was collected in heparinized syringes or by leukapheresis. Peripheral blood mononuclear cells were isolated using Ficoll-Hypaque (Pharmacia). T cell-enriched and T cell-depleted populations were prepared by 'rosetting' with neuraminidase-treated sheep red blood cells as described<sup>24</sup>. T cells were further purified from T cell-enriched cells for the CTL recall assays by removing monocytes, natural killer (NK) cells, and B cells as described<sup>24</sup>. DCs were generated from peripheral blood precursors by culturing T cell-depleted cells for 7 days in the presence of GM-CSF (Immunex, Seattle, Washington) and IL-4 (Schering-Plough, Kenilworth, New Jersey), followed by culturing for 4 days in monocyte conditioned medium<sup>10</sup>.

**Synthetic peptides.** Human PCD peptides were predicted based on anchor residues for HLA A2.1 (ref. 25) and were synthesized for use in cytotoxicity assays (Biosynthesis, Lewisville, Texas). These peptides are (numbering is relative to the full-length mouse cdr2 sequence<sup>8</sup>; GeneBank accession # 1857921): cdr2-1 (KLVPDSLYV; amino acids 273–281), cdr2-2 (SLLEEMFLT; amino acids 289–297), cdr2-3 (QMLQSEHPFV; amino acids 259–268) and cdr2-4 (SLLEEMFLT; amino acids 289–298). The HLA-A2.1-restricted immunodominant peptide derived from the influenza matrix protein MP (GILGFVFTL) served as a control<sup>11</sup>.

**Cytotoxicity assays.** Activated CTLs were detected using T cells as effector cells in a conventional  $\text{Na}^{51}\text{CrO}_4$ -release assay directly after purification. T2 cells (a TAP<sup>-/-</sup>, HLA-A2.1<sup>+</sup>, class II<sup>-</sup> cell line) were 'pulsed' for 1 hour with 1  $\mu\text{M}$  of various peptides, loaded with  $\text{Na}^{51}\text{CrO}_4$  and used as targets<sup>24</sup>. Alternatively, memory CTL responses were stimulated using DCs 'pulsed' for 2–4 hours with 1  $\mu\text{M}$  of various peptides at 25 °C. 'Peptide-pulsed' DCs were then cultured with purified T cells, and after 7 days, responding T cells were assayed for cytolytic activity. Again, T2 cells 'pulsed' with peptide served as targets<sup>24</sup>. In cross-presentation cytotoxicity experiments, HeLa cells were triggered to undergo apoptosis using an ultraviolet B lamp (model# 60UVB; Derma Control, Dolton, Illinois), calibrated to provide 2  $\text{mJ}/\text{cm}^2$  per second (ref. 11). These cells served as a source of cdr2 antigen and were co-cultured with DCs and T cells prepared from PCD patients. After 7 days, responding T cells were assayed for cytolytic activity using 'peptide-pulsed' T2 cells as targets. In all CTL assays, percent cytotoxicity was determined by using the average values of triplicates from experimental wells (E) as compared to average values of spontaneous (S) and total (T) release as follows: % cytotoxicity =  $[(E-S)/(T-S)] \times 100$ . Background lysis is shown in all experiments and ranged from 0% to 3% in the activated and

memory CTLs, and from 5% to 22% in the cross-presentation assays, presumably because of a cross-over reaction between HeLa derived and T2 generated peptides.

**Western blot analysis.** The human cdr2 fusion protein produced from a full-length cDNA (ref. 6) was run on a 10% SDS-PAGE, transferred to nitrocellulose, and blotted against the serum (1:10,000 dilution) and CSF (1:500 dilution) of PCD patients. As a specificity control, serum (1:500 dilution) from a patient with an irrelevant PND (Hu syndrome) was also blotted.

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# Mouse Chromosomal Locations of Nine Genes Encoding Homologs of Human Paraneoplastic Neurologic Disorder Antigens<sup>1</sup>

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The paraneoplastic neurologic disorders (PND) are a rare group of neurologic syndromes that arise when an immune response to systemic tumors expressing neuronal proteins ("onconeural antigens") develops into an autoimmune neuronal degeneration. The use of patient antisera to clone the genes encoding PND antigens has led to new insight into the mechanism of these autoimmune disorders. The tumor antigens can now be grouped into three classes: (1) neuron-specific RNA-binding proteins, (2) nerve terminal vesicle-associated proteins, and (3) cytoplasmic signaling proteins. To understand better the evolutionary relatedness of these genes and to evaluate them as candidates for inherited neurological disorders, we have determined the mouse chromosomal locations of nine of these genes—*Hua*, *Hub*, *Huc*, *Hud*, *Nova1*, *Nova2*, *Natpb*, *Cdr2*, and *Cdr3*. These data suggest that the *Hua*–*Hud* genes arose from gene duplication and dispersion, while the other genes are dispersed in the genome. We also predict the chromosomal locations of these genes in human and discuss the potential of these genes as candidates for uncloned mouse and human mutations.

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## INTRODUCTION

A number of neuron-specific genes have been isolated using autoimmune antisera from patients with paraneoplastic neurologic disorders (PNDs; reviewed in Darnell, 1996). PNDs are believed to be immunologically mediated disorders initiated when tumors, not directly involving the nervous system, ectopically express neuron-specific proteins, termed onconeural antigens. Such tumor antigens become the target of an

effective anti-tumor immune response, which gains access to the nervous system and culminates in autoimmune neuronal degeneration. High-titer antibodies obtained from PND patients have been used to clone a series of target onconeural antigens from human brain cDNA libraries.

A sufficient number of onconeural antigens have been identified to classify them into three groups according to their putative cellular roles (Darnell, 1996). The largest group is the neuron-specific RNA-binding proteins (n-RBPs), in which two distinct gene families have been identified. The first gene family, containing the gene *NOVA1* (Buckanovich *et al.*, 1993) and a second closely related gene *NOVA2* (Darnell *et al.*, unpublished results), was identified using antisera from patients with a PND involving the subcortical motor system. The *Nova* genes encode n-RBPs containing three KH-type RNA-binding domains and are homologous with a number of RNA-binding proteins, including hnRNP-K, FMR-1, the fragile-X mental retardation gene, and several factors that regulate alternative splicing, including the *Drosophila* protein PSI (Siebel *et al.*, 1995), the yeast protein MER-1 (Engbrecht and Roeder, 1990), and the mammalian proteins KSRP and SF1 (Arning *et al.*, 1996; Min *et al.*, 1997). The second gene family of n-RBPs was identified through the cloning of a target antigen termed HuD (Szabo *et al.*, 1991) in a multifocal neurodegenerative PND termed the Hu syndrome. Subsequently several highly related genes were identified by degenerate PCR cloning and cDNA screening from *Xenopus*, mouse, and human libraries (Levine *et al.*, 1993; Good, 1995; Ma *et al.*, 1996; Okano and Darnell, 1996; Sakai *et al.*, 1993). The encoded proteins include Hel-N1 (human), elrB (*Xenopus*), and HuB (mouse); ple21, elrC, and HuC; and HuR, elrA, and HuA (see Okano and Darnell, 1996). All of the Hu genes encode RNA-binding proteins harboring three consensus RRM motifs and share significant sequence homology with *elav*, a *Drosophila* protein essential for neurogenesis, and a lesser but significant homology with *sxl*, a *Drosophila* protein involved in splice-site selection.

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A second group of neuronal antigens includes nerve terminal vesicle-associated proteins. Amphiphysin and glutamic acid decarboxylase are target antigens in stiff-man syndrome (DeCamilli *et al.*, 1993; Solimena *et al.*, 1988), synaptophysin and the  $\beta$ -subunit of the presynaptic calcium channel are target antigens in Lambert-Eaton myasthenic syndrome (Leveque *et al.*, 1992; Rosenfeld *et al.*, 1993), and  $\beta$ -NAP is a target antigen in autoimmune cerebellar degeneration (Newman *et al.*, 1995).

A third group of neuronal antigens includes the cytoplasmic proteins putatively involved in aspects of neuronal signal transduction. This group includes recoverin, a protein involved in cGMP-mediated signal transduction in the retina that is an antigen in paraneoplastic blindness (Polans *et al.*, 1991), and the cerebellar degeneration antigens cdr1, cdr2, and cdr3 (Dropcho and Payne, 1986; Sakai *et al.*, 1990; Fathallah-Shaykh *et al.*, 1991; Corradi *et al.*, 1997). Paraneoplastic cerebellar degeneration arises in patients with breast or ovarian cancer who harbor antisera that recognize a minor (34 kDa) antigen and a major (62 kDa) antigen present in Purkinje neurons. Expression cloning from a cerebellar cDNA library identified the 34-kDa antigen as cdr1 and the 62-kDa antigen as a leucine zipper protein termed cdr62 (Fathallah-Shaykh *et al.*, 1991) or pcd17 (Sakai *et al.*, 1990) and here referred to as cdr2 (Corradi *et al.*, 1997). A cDNA encoding a leucine zipper protein highly related to cdr2 has also been identified from a human HeLa cell library (Fathallah-Shaykh and Posner, unpublished results; GenBank Accession No. L02867); this gene is also expressed in brain and has been termed CDR3 (Corradi *et al.*, 1997).

Given the physiological and structural relatedness among members of the different onconeural families, we have asked what the genomic distributions of members of each of the three groups of antigens are and whether the gene locations of individual family members might correlate with the chromosomal locations of known neurologic disorders in mammals.

## MATERIALS AND METHODS

**Cloning.** Standard molecular techniques were used for cloning and probe generation. The *Hu* and *Nova1* gene sequences used in this study are derived from previously reported mouse homologs of human gene sequences (Buckanovich *et al.*, 1993; Okano and Darnell, 1996). Mouse homologs of NADPTB, NOVA2, CDR2, and CDR3 were obtained by low-stringency screening of a mouse brain cDNA library (Stratagene) using human coding region probes. The human NOVA2 gene was isolated using POMA antisera to screen a human small cell lung cancer library; details of this study, including the complete mouse and human cDNA sequences, are reported elsewhere (Yang, Yin, and Darnell, in preparation).

**Probes.** The probe for *Cdr2* was a 1.5-kb *EcoRI* fragment of mouse cDNA. The probe for *Cdr3* was a 0.8-kb *EcoRI/KpnI* fragment of mouse cDNA containing the 3' coding region and untranslated region (UTR). The probe for *Naptb* was a 0.7-kb *XhoI* fragment of mouse cDNA. The probe for *Nova1* was a 0.7-kb *EcoRI/XhoI* fragment of mouse cDNA. The probe for *Nova2* was a 0.6-kb *EcoRI/XhoI* fragment of mouse cDNA containing the 3' coding region (0.2 kb) and UTR.

The probe for *Hua* was a 0.23-kb *EcoRI* fragment of mouse cDNA containing the 3' UTR. The probe for *Hub* was a 0.252-kb *EcoRI* fragment of mouse cDNA containing the 3' UTR. The probe for *Huc* was a 0.4-kb *EcoRI* fragment of mouse cDNA containing the 3' UTR. The probe for *Hud* was a 0.2-kb *EcoRI* fragment of mouse cDNA containing the 3' UTR.

**Interspecific backcross mapping.** Interspecific backcross progeny were generated by mating (C57BL/6J  $\times$  *Mus spretus*)F<sub>1</sub> females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N<sub>2</sub> mice were used to map the PNDA genes (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.*, 1982). All blots were prepared with Hybond-N<sup>+</sup> membrane (Amersham). Probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a nick-translation labeling kit (Boehringer Mannheim); washing was performed to a final stringency of 1.0–0.25  $\times$  SSCP, 0.1% SDS, at 65°C.

The fragments detected by each of the PNDA probes in restriction enzyme-digested C57BL/6J and *M. spretus* DNAs are shown in Table 1. The presence or the absence of the *M. spretus*-specific fragments was followed in the backcross mice.

The map locations of several other loci used to position the PNDA loci on our interspecific backcross have been previously described. These loci include *Ifa* (Ceci *et al.*, 1989), *Pgm2* (Sutton *et al.*, 1996), and *Cyp4a10* (Bell *et al.*, 1993) on chromosome 4; *Tgfb1* (Dickinson *et al.*, 1990), *Gpi1* (Siracusa *et al.*, 1991), *Igf1r* (Copeland *et al.*, 1992), *Fes* (Copeland *et al.*, 1992), *Lmo1* (Foroni *et al.*, 1992), and *Pkcb* and *Il4r* (Pritchard *et al.*, 1991) on chromosome 7; *Insr*, *Plat* (Ceci *et al.*, 1990), and *Fgfr1* (Kuo *et al.*, 1991) on chromosome 8; *Mmel1* (Shapiro *et al.*, 1992), *Ldlr* (Kingsley *et al.*, 1989), *Epor* (Budarf *et al.*, 1990), and *Aplp2* (von Koch *et al.*, 1995) on chromosome 9; *Ifng*, *Gli* (Justice *et al.*, 1990), and *ErbB3* (Copeland *et al.*, 1995) on chromosome 10; *Pkca* (Buchberg *et al.*, 1989), *Grin2c* (Nagasawa *et al.*, 1996), and *P4hb* (Morishige *et al.*, 1993) on chromosome 11; *Lamb1-1* (Sun *et al.*, 1991), *Hfhbf1* (Avraham *et al.*, 1995), and *Sos2* (Webb *et al.*, 1993) on chromosome 12; and *Mas1* (Cebra-Thomas *et al.*, 1992), *Pim1*, and *H2* (Siracusa *et al.*, 1991) on chromosome 17. Information about these loci can be obtained at <http://www.informatics.jax.org/>.

Recombination distances were calculated as described by Green (1981) using the computer program MapManager. Gene order was determined by minimizing the number of double-recombination events required to explain the allele distribution patterns. The MGD accession number for the data reported here is MGD-JNUM-40255.

## RESULTS AND DISCUSSION

### Mapping Results

The mouse chromosomal locations of nine genes encoding homologs of human paraneoplastic neurological disorder antigens were determined using an interspecific backcross mapping panel derived from crosses of [(C57BL/6J  $\times$  *M. spretus*)F<sub>1</sub>  $\times$  C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2300 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991; Copeland and Jenkins, unpublished observations). C57BL/6J and *M. spretus* DNAs were digested separately with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment polymorphisms (RFLPs) (Table 1) using probes derived from the cDNAs. The strain distribution pattern of each RFLP was then determined by following the presence or absence of the *M. spretus*-specific RFLP in backcross mice.

The mapping results indicate that the PNDA-encod-

TABLE 1

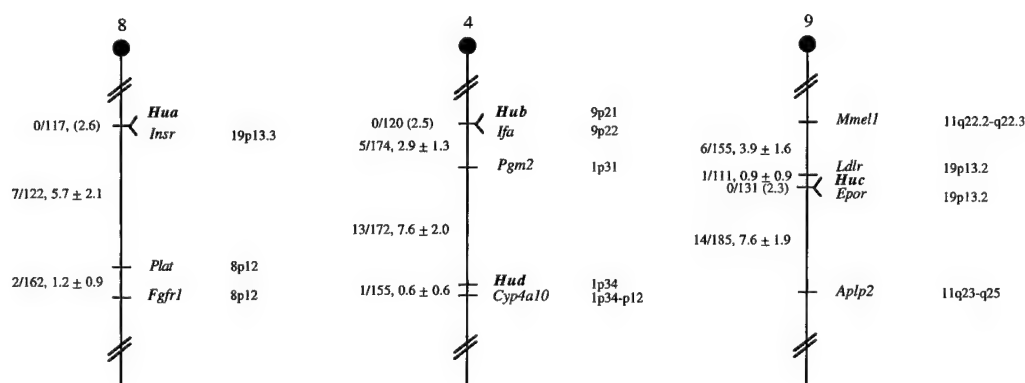
Gene designation	Gene name	Enzyme	C57BL/6J fragment size (kb)	<i>M. spretus</i> fragment size (kb)
<i>Hua</i>	Hu antigen a	<i>HincII</i>	4.6	<u>0.9</u>
<i>Hub</i>	Hu antigen b	<i>PstI</i>	4.0	<u>8.0</u>
<i>Huc</i>	Hu antigen c	<i>PvuII</i>	8.2	<u>7.0</u>
<i>Hud</i>	Hu antigen d	<i>BglII</i>	10.5	<u>7.6</u>
<i>Nova</i>	Ventral neuron-specific protein	<i>XbaI</i>	11.0, 1.4 <sup>a</sup>	11.0, <u>6.2</u> , 1.4 <sup>a</sup>
<i>Nova2-rs1</i>	Ventral neuron-specific protein 2, related sequence 1	<i>HindIII</i>	15.0, 8.8, 7.4, 3.2, 1.7 <sup>a</sup>	<u>6.4</u> , 5.0, 3.4 <sup>a</sup>
<i>Nova2-rs2</i>	Ventral neuron-specific protein 2, related sequence 2	<i>HindIII</i>	15.0, 8.8, 7.4, 3.2, 1.7	6.4, <u>5.0</u> , 3.4
<i>Nova2-rs3</i>	Ventral neuron-specific protein 2, related sequence 3	<i>HindIII</i>	15.0, 8.8, 7.4, 3.2, 1.7	6.4, 5.0, <u>3.4</u>
<i>Naptb</i>	Neuronal adaptin-like protein $\beta$ -subunit	<i>ScaI</i>	13.0, 8.2, 7.1, 5.5, 3.8	<u>17.0</u> , 13.5, 5.5, 3.8
<i>Cdr2</i>	Cerebellar degeneration related 62-kDa protein	<i>BglII</i>	12.0, 4.6	<u>3.3</u>
<i>Cdr3</i>	Cerebellar degeneration related III	<i>XbaI</i>	6.3	<u>3.6</u>

<sup>a</sup> Major bands.

ing genes are dispersed throughout the mouse genome. The Hu antigen loci map to mouse chromosomes 4, 8, and 9 (Fig. 1). On mouse chromosome 4, Hud maps in a region syntenic with human 1p, consistent with its reported human chromosomal location of 1p34 (Muresu *et al.*, 1994). Hub also maps on mouse chromosome 4, 10.5 cM centromeric of Hud, at the junction of the 1p and 9p syntenic regions, consistent with its human map position of 9p21 (Han *et al.*, 1996). Hua and Huc map to mouse chromosomes 8 and 9, respectively; both are in regions syntenic with human chromosome 19p13. Hua is most highly related to the *Drosophila* proteins *elav* and *sxl* (Okano and Darnell, 1996). Hua is 72–73% similar to the other mouse Hu genes, which

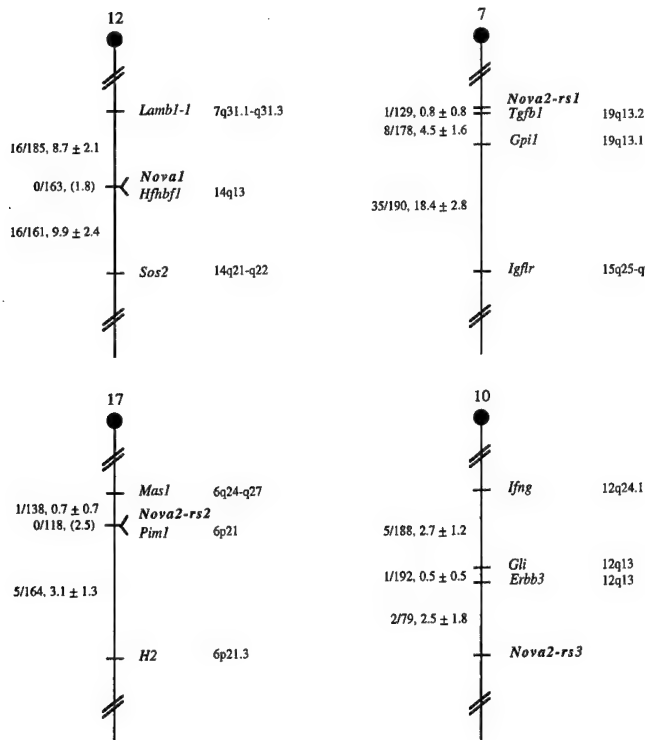
are 80–85% similar to one another. Phylogenetic analysis suggests a duplication of *Hua*, followed by more recent dispersion of the Hu genes. There is evidence for a large 19p13 duplication, given the disposition of members of gene families, including the NFI transcription factors, Jak kinases, Jun protooncogenes, and prostaglandin receptors on 19p13. These gene families have additional members that localize to 1p, indicating that these are paralogous segments that presumably arose by large-scale chromosomal duplication. Interestingly, the four NFI transcription factor genes also localize to 19p13, 1p31, and 9p24 (Qian *et al.*, 1995).

The genes encoding the KH-type RNA-binding proteins Nova-1 and Nova-2 are unlinked to the Hu gene



**FIG. 1.** Murine chromosomal location of Hu genes. Four mouse homologs of PNDA-encoding genes were mapped to mouse chromosomes by interspecific backcross analysis. Partial chromosome linkage maps showing the locations of the PNDA loci (boldface) in relation to linked markers are shown. The number of recombinant  $N_2$  animals over the total number of  $N_2$  animals typed plus the recombination frequencies expressed as genetic distance ( $\pm 1$  standard error) is shown for each pair of loci to the left of each chromosome. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants are found between loci. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution pattern. The positions of loci mapped to human chromosomes are shown to the right of the chromosome maps. References for the map positions of most human loci can be obtained from NCBI/OMIM, a computerized database of human linkage information maintained by the National Center for Biotechnology Information at the National Library of Medicine (<http://www.ncbi.nlm.nih.gov/>).





**FIG. 2.** Murine chromosomal location of *Nova1* and *Nova2* genes. Two mouse homologs of PNDA-encoding genes were mapped to mouse chromosomes by interspecific backcross analysis. Partial chromosome linkage maps showing the locations of the PNDA loci (boldface) in relation to linked markers are shown. The number of recombinant  $N_2$  animals over the total number of  $N_2$  animals typed plus the recombination frequencies expressed as genetic distance in centimorgans ( $\pm 1$  standard error) is shown for each pair of loci to the left of each chromosome. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants are found between loci. The positions of loci mapped to human chromosomes are shown to the right of the chromosome maps.

family (Fig. 2). *Nova1* maps to mouse chromosome 12, while RFLPs detected with the probe for *Nova2* map to mouse chromosomes 7, 10, and 17. Characterization of the *Nova2* gene structure, currently under way, will resolve the relationships among these loci. The map location of *Nova1* predicts that this gene will map to 14q in human. The *Nova2* loci are predicted to map to 19q, 6p, and 12q, respectively.

The leucine zipper protein genes *Cdr2* and *Cdr3* map to mouse chromosomes 7 and 11, respectively (Fig. 3). *Cdr2* maps to mouse chromosome 7 in a region syntenic with its reported human chromosomal location of 16p13.1-p12 (Gress *et al.*, 1992). *Cdr3* is predicted to be on human 17q.

Finally, the neuronal vesicle coat protein gene *Naptb* maps to mouse chromosome 7, in a region syntenic with human chromosome region 15q (Fig. 3).

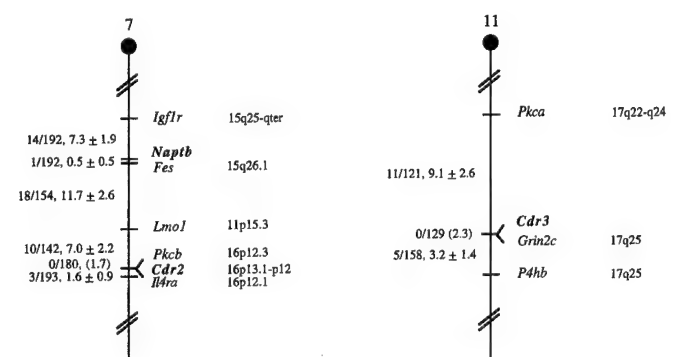
#### Linkage to Known Neurological Mutations

Since the PNDs lead to discrete neurologic disorders, mutations in the target antigens may lead to syndromes with similar features. Several of the current chromo-

somal localizations of PNDA genes suggest candidate mouse or human mutations that might result from a PNDA gene mutation; these are listed in Table 2.

*Hua* maps near two mouse neurological mutations on chromosome 8, nervous (*nr*) and motor neuron degeneration (*mnd*). Analysis of a backcross in which *nr* is segregating indicates that *Hua* can be excluded as a candidate (Fletcher, Copeland, and Jenkins, unpublished observations). *Hua* remains a candidate for motor neuron degeneration, which results in upper and lower spinal motor neuron degeneration and ataxia. *Hub* maps to chromosome 4 near meander tail, but analysis of a backcross in which meander tail is segregating has excluded *Hub* (Fletcher, Copeland, and Jenkins, unpublished observations). *Huc* does not map near any neurological mutations in mouse. *Hud* maps near clasper, whose phenotype of hobbled gait and generalized tremor indicates a neurological origin.

*Hua* and *Huc* are predicted to map on 19p13 in human, where Cayman-type cerebellar ataxia has recently been localized. The robust expression of *Huc* in the cerebellum and neocortex, particularly its exclusive expression in Purkinje neurons (relative to other *Hu* genes; Okano and Darnell, 1996), suggests that *Huc* is an excellent candidate gene for Cayman-type cerebellar degeneration, which involves predominantly cerebellar dysfunction as well as some cortical dysfunction (psychomotor retardation). Given that the major symptom present in the *Hu* syndromes is a neuronal sensory neuropathy (Dalmau *et al.*, 1991), it is of interest that the *Hud* gene maps near the CMT2A locus. CMT2A is a neuronal form of neuropathy and thus is distinct from the peripheral (axonal, demyelinating) types of CMT neuropathy. Further characterization of these loci will be required to determine if mutations in *Hu* genes underlie these syndromes.



**FIG. 3.** Murine chromosomal location of *Cdr* and adaptin-like genes. Three mouse homologs of PNDA-encoding genes were mapped to mouse chromosomes by interspecific backcross analysis. Partial chromosome linkage maps showing the locations of the PNDA loci (boldface) in relation to linked markers are shown. The number of recombinant  $N_2$  animals over the total number of  $N_2$  animals typed plus the recombination frequencies expressed as genetic distance in centimorgans ( $\pm 1$  standard error) is shown for each pair of loci to the left of each chromosome. The upper 95% confidence limit of the recombination distance is given in parentheses when no recombinants are found between loci. The positions of loci mapped to human chromosomes are shown to the right of the chromosome maps.

TABLE 2

Locus	Mouse chromosome	Candidate mouse genes	Predicted human locus (known)	Candidate human genes
<i>Hua</i>	8	<i>nr, mnd</i>	19p13	ATCAY
<i>Hub</i>	4	<i>mea</i>	(9p21)	—
<i>Huc</i>	9	—	19p13	ATCAY
<i>Hud</i>	4	<i>cla</i>	(1p34)	CMT2A
<i>Nova1</i>	12	—	14q	Spastic paraplegia 3
<i>Nova2-rs1</i>	10	—	19q	—
<i>Nova2-rs2</i>	7	—	6p	—
<i>Nova2-rs3</i>	17	—	12q	—
<i>Naptb</i>	7	<i>asp3, twt</i>	15q	Spastic paraplegia 6
<i>Cdr2</i>	7	—	(16p13.1-p12)	—
<i>Cdr3</i>	11	<i>tn, cod</i>	17q	—

The *Nova1* gene is predicted to map in the region of spastic paraplegia on 14q, an observation of interest since in both disorders there is a similar dysfunction of spinal motor neurons. In particular, the myoclonus and ataxia, characteristic of POMA (in which *Nova-1* is targeted), are similar to the motor spasticity and ataxia seen in spastic paraplegia. Moreover, patients with spastic paraplegia have no evidence of a peripheral sensory or motor nerve defect, consistent with the restricted expression of NOVA1 to the central nervous system (Buckanovich *et al.*, 1993). However, the lack of cranial nerve signs in spastic paraplegia would be unexpected for a NOVA1 mutation, given the abundance of NOVA1 expression in the brain stem (Buckanovich *et al.*, 1993, 1996) and the cranial nerve defect (opsoclonus) present in POMA patients (Darnell, 1994). Evaluation of the *Nova2* gene will be performed after the structures of the loci identified by the *Nova2* probe are determined.

$\beta$ -NAP was identified as a target antigen in a patient with pan-cerebellar dysfunction. Subsequent studies have suggested that  $\beta$ -NAP is part of a neuron-specific vesicle coat protein complex and may be involved in the budding of vesicles in the neuronal cell soma that are sorted to the axonal nerve terminus (Newman *et al.*, 1995; Simpson *et al.*, 1996). Candidate mouse mutations mapping to the region of the *Naptb* gene include audiogenic seizure prone 3 (*asp3*) and twister (*twt*). The *asp3* mutation results in generalized seizures following loud noises, a phenotype similar to the human syndrome of myoclonic epilepsy, which is generally found in conditions associated with a diffusely abnormal cortex (Andermann and Andermann, 1986), while twister mice show a nonspecific neurologic phenotype of head tucking and circling. While mutations in *Naptb*, which is expressed in all postmitotic neurons (Newman *et al.*, 1995), might correlate with widespread neuronal dysfunction, such a phenotype does not clearly correlate with the cerebellar degeneration seen in patients with autoimmunity directed against  $\beta$ -NAP protein (Darnell

*et al.*, 1991). The absence of cerebellar degeneration likewise suggests that *Naptb* is an unlikely candidate for spastic paraplegia 6.

The *Cdr2* locus is not near any neurological loci in the mouse. However, *Cdr3* maps near the mouse mutations teetering and cerebellar outflow degeneration. Given that *Cdr3* is abundantly expressed in Purkinje neurons (Corradi *et al.*, 1997; Darnell *et al.*, unpublished data), it is an excellent candidate for these mutations, which principally affect the cerebellum and associated mid-brain nuclei. Preliminary analysis of Southern blots does not show any alteration of *Cdr3* in teetering mice, and further evaluation is ongoing.

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## The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia

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**ABSTRACT** Paraneoplastic opsoclonus myoclonus ataxia (POMA) is a neurologic disorder thought to be mediated by an autoimmune attack against onconeural disease antigens that are expressed by gynecologic or lung tumors and by neurons. One POMA disease antigen, termed Nova-1, has been identified as a neuron-specific KH-type RNA-binding protein. Nova-1 expression is restricted to specific regions of the central nervous system, primarily the hindbrain and ventral spinal cord, which correlate with the predominantly motor symptoms in POMA. However, POMA antisera recognize antigens that are widely expressed in both caudal and rostral regions of the central nervous system, and some patients develop cognitive symptoms. We have used POMA antisera to clone a cDNA encoding a second POMA disease antigen termed Nova-2. Nova-2 is closely related to Nova-1, and is expressed at high levels in neurons during development and in adulthood, and at lower levels in the adult lung. In the postnatal mouse brain, Nova-2 is expressed in a pattern that is largely reciprocal with Nova-1, including high levels of Nova-2 expression in the neocortex and hippocampus. Functional characterization of Nova-2 in RNA selection and nitrocellulose filter-binding assays reveals that Nova-2 binds RNA with high affinity and with sequence specificity that differs from Nova-1. Our results demonstrate that the immune response in POMA targets a family of highly related sequence-specific neuronal RNA-binding proteins. The expression pattern of the Nova-2 protein is likely to underlie the development of cognitive deficits in some POMA patients.

The paraneoplastic neurologic diseases (PNDs) are an unusual group of diseases at the intersection of neurobiology, immunology, and oncology. Patients with PNDs harbor systemic tumors and develop immune responses against onconeural antigens that are expressed both by their tumors and by neurons (for review, see refs. 1 and 2). The presence of high-titer antibodies in patient sera has allowed the identification and characterization of PND antigens and has provided a unique approach toward understanding the expression and function of these proteins in discrete regions of the brain. For example, the CAR antibody, associated with paraneoplastic blindness and small cell lung cancer (SCLCa), was used to identify recoverin, a protein involved in receptor signaling in the photoreceptor (3, 4), the Yo antibody, associated with paraneoplastic cerebellar degeneration, was used to identify cdr2, a novel leucine-zipper protein expressed in cerebellar Purkinje neurons (5, 6), and the Nb antibody, found in a patient with cerebellar degeneration, was used to identify a neuron-specific, adaptin-like protein called  $\beta$ -NAP (7, 8).

Two distinct families of neuronal RNA-binding proteins, Nova and Hu, have also been identified as PND target antigens (for review, see ref. 2). The Hu proteins were identified in a

PND associated with SCLCa (9, 10). This family contains at least four highly related RNA recognition motif (RRM)-type RNA-binding protein members and shares strong homologies with the *Drosophila* neurogenic gene *elav* and the splicing and translational control factor *sxl* (11–14). Within the Hu family, there exists a high degree of complexity, in part because of distinct developmental expression patterns of the multiple family members (15). The Nova-1 protein was identified in paraneoplastic opsoclonus myoclonus ataxia (POMA), which is associated with breast cancer, fallopian cancer, and SCLCa, and is characterized primarily by dysfunction of the motor nervous system (16, 17). The Nova-1 cDNA encodes a sequence-specific K homology (KH)-type RNA-binding protein whose RNA-binding ability can be abrogated *in vitro* by POMA disease antiserum (17–19). Nova-1 expression is restricted to subcortical structures in the central nervous system (CNS), both during mouse development and in the adult brain (17, 18). However, POMA antisera are reactive against all neurons in the mouse and human CNS by immunohistochemistry (16, 17, 20, 21), and POMA antibodies, affinity-purified with Nova-1 fusion protein (NFP), recognize multiple immunoreactive bands in mouse brain extracts at 50–55 kDa and 70–80 kDa (17), suggesting that additional Nova-1-related POMA antigens may exist. Clinical reports have documented progressive neurological deficits in some POMA patients. In up to 58% of patients, multifocal neurological deficits such as encephalopathy and dementia with cerebral atrophy are seen, suggesting involvement of rostral brain regions that do not express Nova-1 (refs. 16 and 22; for review, see ref. 23).

In the present study, we used POMA antisera to expression clone a second Nova family member, termed Nova-2. Nova-2 is a neuronal KH-type RNA-binding protein expressed in a broader CNS distribution than Nova-1. We have compared the expression patterns and RNA-binding properties of Nova-1 and Nova-2 and conclude that the Nova-2 protein is a PND antigen that is likely to function in neuronal RNA metabolism. Furthermore, the expression pattern of Nova-2 may underlie the neurologic dysfunction in POMA patients who develop encephalopathy and dementia.

### MATERIALS AND METHODS

**Library Screening and Cloning of Nova-2.** A human SCLCa cDNA expression library (Stratagene) was screened as described (17), by using POMA antiserum. Full-length Nova-2

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: PND, paraneoplastic neurologic disease; SCLCa, small cell lung cancer; RRM, RNA recognition motif; POMA, paraneoplastic opsoclonus myoclonus ataxia; KH, K homology; CNS, central nervous system; NFP, Nova-1 fusion protein; N2FP, Nova-2 fusion protein; N2Ab, Nova-2-specific antibody; NLS, nuclear localization signal.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. AF083898).

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clones were identified by screening human hippocampal, mouse adult brain, and mouse E12.5 cDNA libraries (Stratagene).

**Production of Fusion Proteins.** Full-length T7 epitope-tagged and histidine-tagged NFP was produced as described (18). Full-length T7 epitope-tagged and histidine-tagged Nova-2 fusion protein (N2FP) was produced in the pET21a vector (Novagen).

**Western Blot Analysis.** Proteins were separated by SDS/PAGE, transferred to nitrocellulose, and probed with patient and control human antisera. Blots were developed with horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (Amersham).

**Northern Blot Analysis.** Total mouse RNA was obtained by using a modified guanidine-acid phenol protocol (24), separated on a formaldehyde/agarose gel, transferred to nylon (Biodyne Electronics, Santa Monica, CA; Pall), and probed with a 310-bp radiolabeled fragment from the Nova-2 coding region in an aqueous solution [ $6\times$  standard saline citrate (SSC) ( $1\times$  SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7);  $2\times$  Denhardt's solution [ $1\times$  Denhardt's 0.02% polyvinylpyrrolidone/0.02% Ficoll (Pharmacia)/0.02% BSA], 0.1% SDS, 100  $\mu$ g/ml denatured salmon sperm DNA]. Autoradiograms were exposed with Kodak MS film at  $-70^{\circ}\text{C}$ .

**In Situ Hybridization.** *In situ* hybridization was performed as described (15). Frozen sections (12  $\mu$ m) were fixed with 4% paraformaldehyde. Sense and antisense RNA probes (310 nt) from the Nova-2 coding region were transcribed *in vitro* with T7 RNA polymerase and  $^{33}\text{P}$ -UTP. Slides were hybridized at  $55^{\circ}\text{C}$  for 2 days with  $1\times 10^6$  cpm-labeled probe per section in 50% formamide hybridization solution. Slides were dipped in Kodak NTB2 emulsion, exposed for 10–21 days, developed, and counterstained with cresyl violet.

**Nova-2-Specific Antipeptide Antibody.** A peptide was synthesized corresponding to the predicted Nova-2 amino acids 392–405 (GGFLTAEKLAESA). Rabbits were immunized with peptide and methylated BSA, with adjuvant. Nova-2 specific antibody (N2Ab) was affinity-purified with covalently bound N2FP beads; specific antibody was eluted with 0.2 M glycine (pH 3.0), neutralized to pH 7.4, and dialyzed against PBS before use.

**Immunoprecipitation.** Mouse embryo extracts were prepared by fresh tissue homogenization in ice-cold NET-2 buffer (150 mM NaCl/50 mM Tris-HCl, pH 7.5/0.1% Nonidet P-40), sonication, and centrifugation at  $18,000\times g$  for 15 minutes. Supernatants were precleared with protein A Sepharose; antibody binding was carried out for 4 hours, and immunoprecipitation pellets were washed in RIPA buffer (150 mM NaCl/50 mM Tris-Cl, pH 7.5/1% Nonidet P-40/0.5% deoxycholate/0.1% SDS) and analyzed by Western blot analysis.

**Immunohistochemical Analysis.** Tissues from mice perfused with Bouin's solution (Sigma) were postfixed in fresh Bouin's solution and 70% ethanol and were paraffin embedded. Sections (14  $\mu$ m) were blocked with normal goat serum and  $\text{H}_2\text{O}_2$ , were incubated with primary antibody for 2 days at  $4^{\circ}\text{C}$ , then incubated with biotinylated secondary antibody. Signal was intensified with Vectastain (Vector Laboratories) and developed in 0.5 mg/ml diaminobenzidine (Sigma).

**Ribohomopolymer Assays.** N2FP (500 ng) and 500 ng control protein were incubated with ribohomopolymer beads (Sigma) in RHPA buffer (10 mM Tris, pH 7.4/2.5 mM  $\text{MgCl}_2$ /0.5% Triton X-100) with the indicated NaCl concentration and 1 mg/ml heparin in a volume of 500  $\mu$ l. Binding occurred at  $4^{\circ}\text{C}$  for 10 minutes, reactions were washed five times in RHPA buffer with the indicated NaCl concentration, boiled in SDS buffer, and run on Western blots.

**RNA Selection Assay.** RNA selection experiments were performed essentially as described (19). For each selection round, radiolabeled RNA was transcribed from an *in vitro* synthesized oligonucleotide template library with an internal

52-bp random sequence and estimated complexity of  $2.1\times 10^{14}$ . Gel-purified RNA was applied to a precolumn to adsorb nonspecifically bound RNAs, then applied to a N2FP nickel affinity column in selection buffer [20 mM Tris-HCl, pH 7.6/1 mM  $\text{MgCl}_2$ /50 mM imidazole, and either 0.5 M LiCl (experiment #1) or 0.3 M LiCl (experiment #2)]. After being washed in 15–30 column volumes of selection buffer, protein and RNA were coeluted by the addition of 1.0 M imidazole in selection buffer. RNA was extracted in phenol/0.5% SDS at  $50^{\circ}\text{C}$ , ethanol precipitated, reverse transcribed, PCR amplified, and RNA was transcribed for RNA selection.

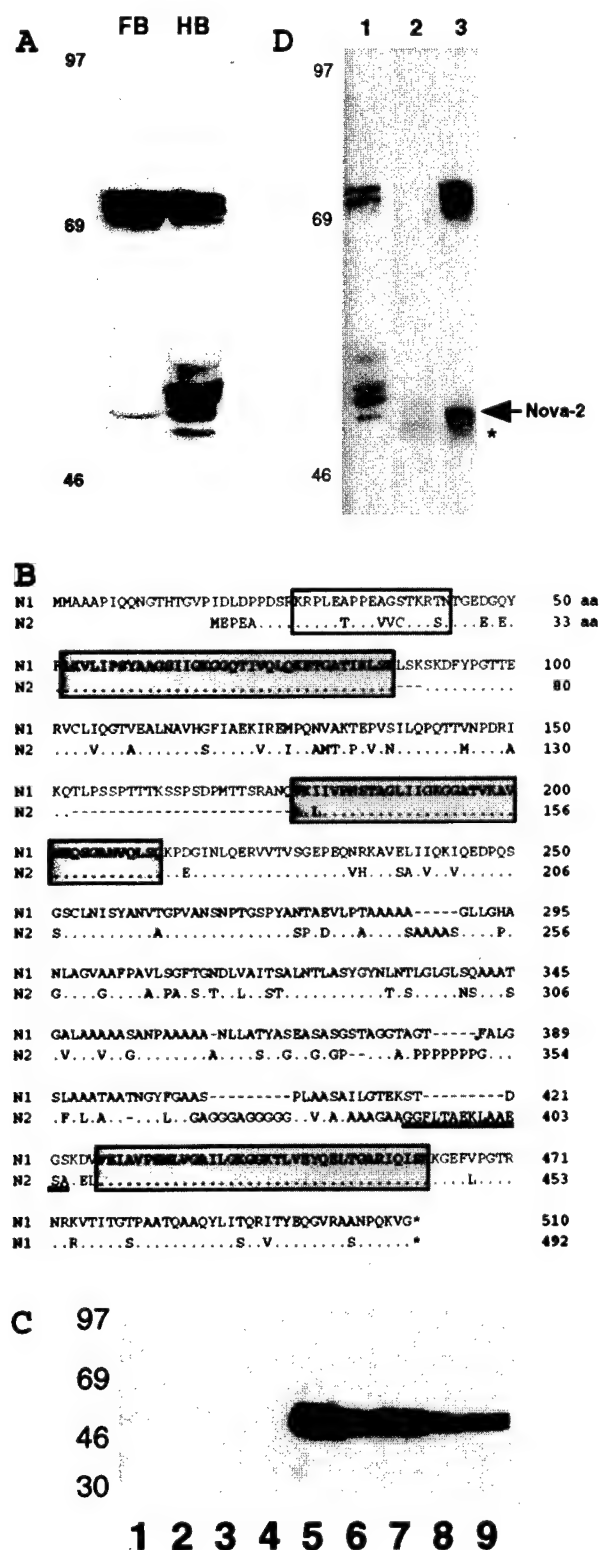
**Filter-Binding Assays.** Filter binding by using purified NFP and N2FP was performed as described (19). Fusion proteins were combined with radiolabeled RNA in 50  $\mu$ l of binding buffer [200 mM KOAc/50 mM Tris-OAc, pH 7.7/5 mM  $\text{Mg}(\text{OAc})_2$ ]. After room-temperature incubation, samples were filtered through nitrocellulose, were washed with binding buffer, and retained counts were determined in a scintillation counter.

## RESULTS

**Cloning of a Nova Family Member.** *In situ* hybridization demonstrated that Nova-1 expression is restricted to the hypothalamus, ventral midbrain, hindbrain, and spinal cord (17, 18). To explore the possibility of additional Nova-like proteins in cortical brain regions, we performed Western blot analysis of mouse P0 forebrain vs. hindbrain with POMA disease antiserum (Fig. 1A). Several immunoreactive antigens of 70–75 kDa and a single antigen of 50 kDa are selectively expressed in forebrain, whereas these and additional antigens of 50–55 kDa are present in the hindbrain. The size and distribution of the 50- to 55-kDa hindbrain bands is consistent with the previous characterization of Nova-1. However, because Nova-1 is not expressed in forebrain, and POMA antibodies affinity purified with NFP also recognize these forebrain antigens (ref. 17 and data not shown), the data in Fig. 1A suggest the expression of a Nova-related antigen in forebrain.

Because the full complement of Nova immunoreactive bands were expressed in a SCLCa cell line (data not shown), we used POMA antiserum to screen a SCLCa expression library. This screen identified a cDNA encoding an immunoreactive protein related to Nova-1, which we term Nova-2 (Fig. 1B). To determine whether Nova-2 fusion protein (N2FP) can be recognized by antisera from different POMA patients, a full-length recombinant N2FP was produced, and N2FP strips were immunoblotted with POMA or control antisera (Fig. 1C). N2FP was recognized at high titers by 5 of 5 different POMA sera, while all control sera were negative, demonstrating that the Nova-2 cDNA encodes a POMA disease antigen.

The human Nova-2 cDNA contains 1,476 nts of ORF (data not shown), encoding 492 amino acids and predicting a protein of 48.9 kDa. Nova-2 is very closely related to Nova-1, with overall 75% amino acid identity and 85% amino acid homology (Fig. 1B). In the regions of the three KH domains, the amino acid sequence is 98% identical to Nova-1, suggesting that the proteins may share similar RNA-binding characteristics. Nova-1 and Nova-2 share significant homology in the regions of the KH domains to other KH-type RNA-binding proteins (data not shown). In addition, both Nova proteins contain a near-match to the consensus bipartite nuclear localization signal (NLS) (25) positioned in the N terminus before the first KH domain (Fig. 1B); this putative NLS is also an 8/17 (Nova-2) or 7/17 (Nova-1) identical amino acid match to a functional N-terminal NLS identified in heterogeneous nuclear RNP-K (26). Nova-2 is also highly conserved across species, as the mouse- and human-predicted proteins are 99% identical (data not shown).



**Fig. 1.** Identification of Nova-2, a PND antigen. (A) Western blot analysis of POMA antigens in P0 mouse brain. Total protein extracts (50  $\mu$ g per lane) from P0 mouse forebrain (FB) or hindbrain (HB) were analyzed by Western blot analysis with POMA antiserum. Molecular mass markers are indicated on the left (kDa). (B) The full-length human Nova-2 amino acid sequence is shown compared with Nova-1. The Nova-1 sequence includes mini-exon LSK (aa 88–90) and alternatively spliced Exon H (aa 153–176). KH domains are boxed in gray. A potential NLS is boxed in white. The peptide sequence used to generate the Nova-2-specific antibody N2Ab (aa 392–405) is underlined. Dashes indicate gaps inserted to align the sequences, dots indicate identity, and asterisks indicate predicted stop codons. (C) The N2FP is recognized by 5 of 5 POMA patient antisera. Western blots

The spacer region between the second and third KH domains contains the least homology between Nova-1 and Nova-2, with 59% amino acid identity and 74% homology (Fig. 1B). The Nova-2 amino acid sequence in this region contains several long stretches of alanine and/or glycine residues and a short stretch of proline residues, while the Nova-1 amino acid sequence contains shorter stretches of alanine and/or glycine and does not contain a proline-rich region. We used a unique 14 amino acid-peptide sequence from the Nova-2 spacer region (Fig. 1B) to develop an antipeptide antibody termed N2Ab that completely distinguished between purified N2FP and NFP (data not shown). Immunoprecipitation with N2Ab from mouse brain extracts demonstrated specific immunoreactivity against the subset of Nova proteins that are expressed in mouse forebrain (75 kDa and a single band of 50 kDa; Fig. 1A, D).

**Expression Analysis of Nova-2.** We examined the expression of Nova antigens in adult mouse tissues by Western blot analysis using POMA antiserum. Immunoreactive Nova proteins were expressed in brain, at significantly lower levels in lung, and were absent in other tissues (Fig. 2A). The set of proteins recognized at 75 kDa and a single band at 50 kDa correspond to the set of Nova-2 antigens defined with the antipeptide antibody N2Ab (Fig. 1C).

To confirm Nova-2 expression in brain and lung, a Nova-2 probe was used on a tissue of Northern blot of mouse total RNA (Fig. 2B). Three Nova-2 transcripts were identified in adult mouse brain at 0.6, 3.0, and  $\geq 9$  kb; the two larger transcripts were also detectable in lung at significantly lower levels. Cross-hybridization to the 4.7-kb Nova-1 transcript (17) was not seen. The Nova-2 transcripts were not detected in kidney, spleen, or heart. The 3-kb band is consistent with the sequence in Fig. 1B, and the 0.6-kb band is consistent with a potential alternatively spliced Nova-2 transcript identified by screening a mouse adult brain cDNA library (data not shown). The 9-kb band has not been identified but was present in both total and poly(A)<sup>+</sup> RNA (data not shown), suggesting that it is not pre-mRNA and is likely instead to represent either an uncharacterized Nova-2 alternatively spliced RNA or a transcript from a highly related gene (see discussion).

To further examine Nova-2 mRNA expression, a Nova-2 riboprobe was used for *in situ* hybridization analysis (Fig. 2C–D). In the E14 mouse embryo, Nova-2 transcripts were detected throughout the entire CNS, with no detectable expression in the peripheral nervous system or in nonneuronal tissues, including lung. At P0, Nova-2 transcripts were detected specifically in the CNS in a widespread pattern, with regional differences in intensity. Highest levels of expression were in the cortex, olfactory bulb, thalamus, inferior colliculus, inferior olive, and the internal and external granule cell layers of the cerebellum, and lowest levels of expression were in the brainstem. A similar pattern of expression was seen in the adult brain at lower levels (data not shown).

with recombinant N2FP (500 ng per strip) were immunoblotted with POMA antisera (lanes 5–9, serum from five different POMA patients at 1:200, 1:250, 1:250, 1:1000, 1:2000), non-POMA paraneoplastic antisera (lane 3, Yo patient serum at 1:200; lane 4, Nb patient serum at 1:200), or normal human control sera (lane 1, 1:50; lane 2, 1:200). Molecular mass markers are indicated on the left (kDa). (D) The antipeptide antibody N2Ab immunoprecipitates a subset of Nova proteins from mouse brain. Immunoprecipitations from E17 mouse brain extracts were performed with affinity-purified N2Ab (lane 3) or a control affinity-purified rabbit antibody (lane 2). Immunoprecipitates were analyzed for Nova proteins by immunoblotting with POMA antiserum. Extract (50  $\mu$ g) was loaded (lane 1) to demonstrate the Nova proteins in the starting material. Molecular mass markers are indicated on the left (kDa); the Nova-2 50-kDa band (arrowhead) and IgG bands (asterisk) are indicated on the right.

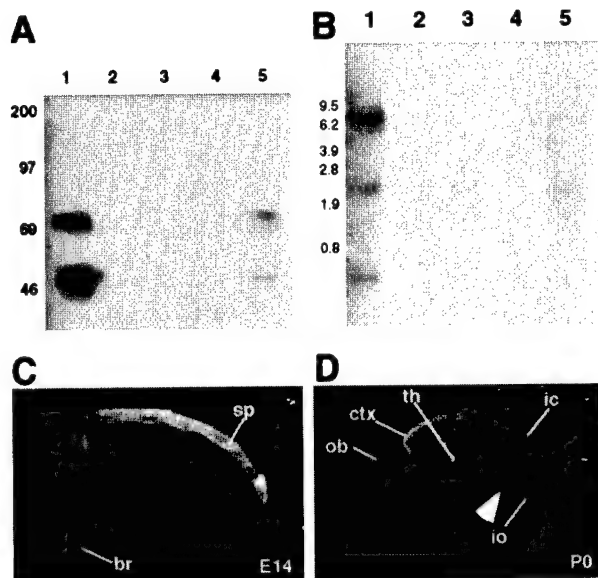


FIG. 2. Expression analysis of Nova-2 in mouse. (A) Nova tissue Western blot. Total protein (50  $\mu$ g per lane) from adult mouse tissues was immunoblotted with POMA antiserum. Lanes: 1, brain; 2, kidney; 3, heart; 4, spleen; 5, lung. Molecular mass markers are indicated on the left (kDa). (B) Nova-2 tissue Northern blot. Total RNA (20  $\mu$ g per lane) was isolated from adult mouse tissues and hybridized on a Northern blot with a Nova-2 specific probe. Lanes: 1, brain; 2, kidney; 3, heart; 4, spleen; 5, lung. Molecular weight markers are indicated on the left (kb). The blot was stripped and reprobed for actin to demonstrate RNA integrity (data not shown). (C and D) Nova-2 transcripts in the mouse embryo and perinatal mouse. Sagittal sections (12  $\mu$ m) of E14 embryo (C) or P0 (D) mouse were hybridized with a  $^{33}$ P-radiolabeled Nova-2 riboprobe and imaged with darkfield microscopy (positive signal is white). At E14, Nova-2 mRNA is detected throughout the CNS, including brain (br) and spinal cord (sp). At P0, Nova-2 transcripts are most abundant in the cortex (ctx), with strong signal also in olfactory bulb (ob), thalamus (th), inferior colliculus (ic), and inferior olive (io). Signal is relatively weak in the brainstem (arrowhead). No signal was seen with control sense riboprobes (data not shown).

Using the Nova-2-specific antibody N2Ab, we carried out immunohistochemical analyses of the Nova-2 protein expression pattern (Fig. 3). N2Ab immunoreactivity at E14 and P0 corresponded precisely with the expression of Nova-2 mRNA defined by *in situ* hybridization (Fig. 2 C–D), and immunoreactivity in the CNS was completely blocked by preincubation of N2Ab with the peptide against which it was generated (data not shown).

To more directly compare Nova-1 and Nova-2 expression, we performed serial-section immunohistochemistry using either N2Ab or POMA antiserum (using fixation conditions that detect primarily Nova-1 protein; ref. 17). The patterns of Nova-1 and Nova-2 immunoreactivity in the P0 CNS were largely reciprocal (Fig. 3). The highest levels of Nova-2 immunoreactivity were detected in CNS regions that express little or no Nova-1, such as neocortex, thalamus, inferior colliculus, inferior olivary nuclei, and the external granule cell layers of the cerebellum. Conversely, lower levels of Nova-2 immunoreactivity were detected in regions of the CNS overlapping with high levels of Nova-1 expression, including the ventral midbrain, hindbrain, and spinal cord, although some neurons did express significant levels of both Nova-1 and Nova-2, including the large motor neurons of the ventral spinal cord (Fig. 3).

**Nova-2 Is a Sequence-Specific RNA-Binding Protein.** Nova-1 has been characterized as a sequence-specific RNA-binding protein by ribohomopolymer binding assays and RNA selection (18, 19). As an initial comparison of the RNA-binding characteristics of Nova-1 and Nova-2, we performed *in*

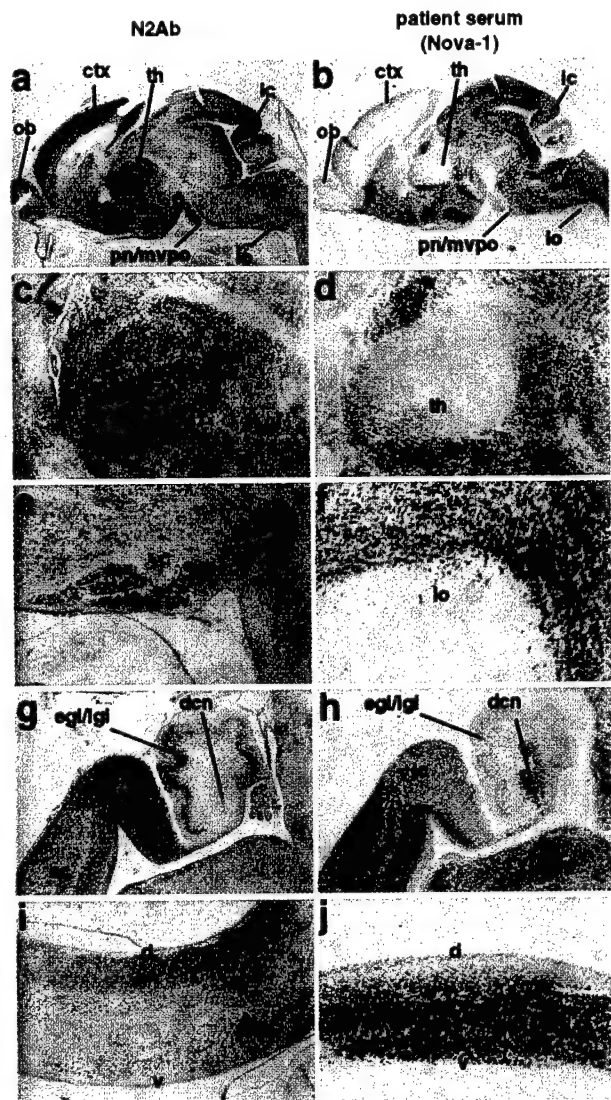
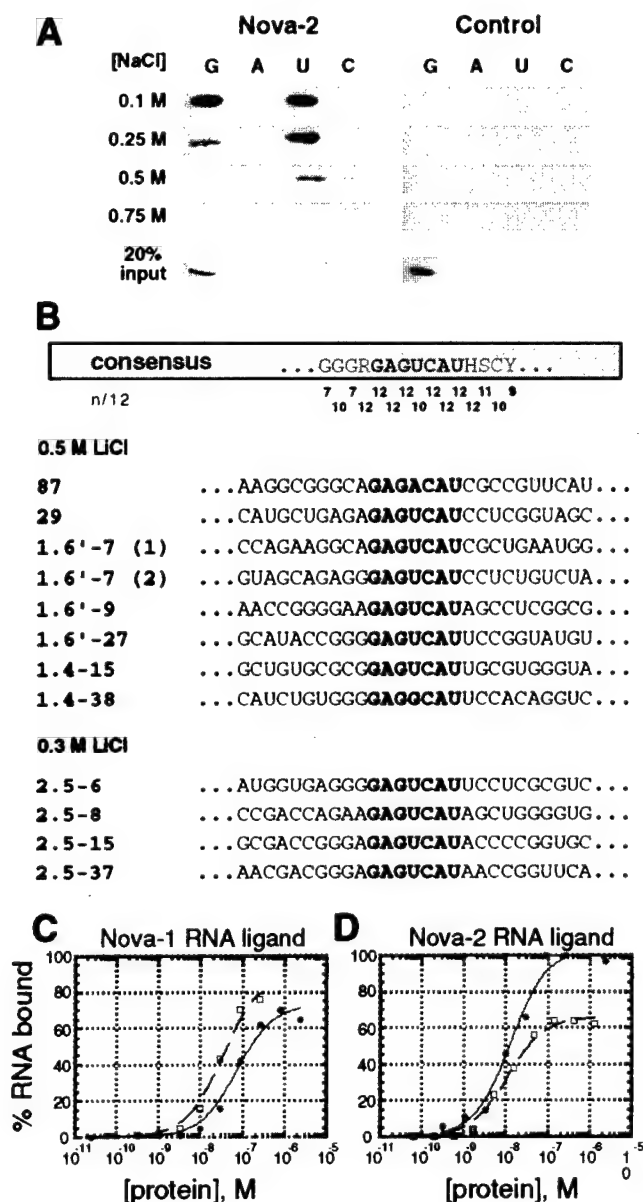


FIG. 3. Comparison of Nova-2 expression with Nova-1 at P0. Reciprocal patterns of Nova-2 and Nova-1 protein expression at P0. Nova-2 immunoreactivity in sagittal sections of a P0 mouse brain was assessed with affinity-purified N2Ab (a, c, e, g, i), and compared in serial sections with POMA antiserum under fixation conditions previously demonstrated to preferentially detect the Nova-1 protein (ref. 17; b, d, f, h, j). Whole brain (a, b), and higher magnification views comparing Nova-1 and Nova-2 expression showing the thalamus [th; (c, d)], inferior olive [io; (e, f)], superior colliculus (sc), inferior colliculus (ic), external and internal granule cell layers of the cerebellum (egl/igl), and deep cerebellar nuclei [dcn; (g, h)], and dorsal (d) and ventral (v) spinal cord (i, j). Arrowhead indicates the large motor neurons in the ventral spinal cord (i, j), where moderate Nova-2 and strong Nova-1 expression is overlapping. Olfactory bulb (ob), cortex (ctx), and pontine nuclei/medioventral periolivary nucleus (pn/mvpo) are also shown.

*vitro* ribohomopolymer binding assays and RNA selection with Nova-2. Recombinant T7 epitope-tagged N2FP bound avidly to riboG and riboU in up to 0.5 M NaCl, while a control protein showed no detectable binding at any salt concentration (Fig. 4A). These results demonstrate that Nova-2 is an RNA-binding protein.

To identify specific RNA sequences that bind Nova-2, we performed affinity elution-based RNA selection with N2FP in two independent selection experiments. We used an *in vitro*-synthesized 52-randomer template pool with an estimated complexity of  $2.1 \times 10^{14}$  (19). In both experiments, a Nova-2 consensus binding sequence was identified that contained a core GAGUCAU motif (Fig. 4B), demonstrating that Nova-2





**FIG. 4.** Nova-2 is an RNA-binding protein. (A) Analysis of Nova-2 RNA-binding ability to ribohomopolymer RNA in varying salt concentrations. N2FP (5  $\mu$ g) or an irrelevant T7 epitope-tagged and histidine-tagged control protein was incubated with poly(G), poly(A), poly(U), and poly(C)-Sepharose ribohomopolymer beads in either 0.1 M, 0.25 M, 0.5 M, or 0.75 M NaCl. After being washed, protein bound to the ribohomopolymers was analyzed by Western blot analysis with an anti-T7 antibody (Novagen). Twenty percent of the input protein is shown (Lower). (B) Affinity elution-based RNA selection identifies a Nova-2 consensus binding sequence. Alignment of 12 Nova-2 RNA ligands selected in two independent Nova-2 selection experiments (in 0.5 and 0.3 M LiCl) is shown. A consensus sequence is shown at the top, with the GAGUCAU motif highlighted. The match to the consensus, scored as the number of exact matches of 12 sequences (n/12), is shown for each position. Bases in RNA ligands with identity to the GAGUCAU motif are also shown highlighted. (C and D). Direct comparisons of Nova-1 and Nova-2 binding to selected RNA ligands. Nitrocellulose filter binding assays were performed to examine Nova-1 (open squares) and Nova-2 (solid circles) binding to RNA ligands mSB2 (C), a 21-nt RNA containing the Nova-1 consensus binding motif [UCAU(N)<sub>0-2</sub>]<sub>3</sub> (19); and 87 (D), a 96-nt RNA ligand isolated by Nova-2 RNA selection (Fig. 4B). RNA ligand mSB2 bound to Nova-1 and Nova-2 with calculated  $K_d$  values of  $33 \pm 5$  nM and  $73 \pm 13$  nM, respectively (calculated by least squares fit; ref. 33). Similar results were found with the 96 nt SB2 Nova-1 RNA ligand (data not shown). RNA ligand 87 bound to Nova-1 and Nova-2 with calculated  $K_d$  values of  $13 \pm 2.5$  nM and  $11 \pm 1.5$  nM, respectively. Maximal

is a sequence-specific RNA-binding protein. This sequence is similar to, but distinct from, the RNA consensus-binding sequence identified by Nova-1 RNA selection, which consists of the sequence [UCAU(N)<sub>0-2</sub>]<sub>3</sub> (19). In addition, we note that the Nova-2-selected RNA ligands are predicted to form a stem-loop structure around the GAGUCAU motif, with a sequence preference for purines in the 5' and pyrimidines in the 3' half of the predicted stem (data not shown). We assessed the binding of Nova-1 and Nova-2 to selected Nova-1 or Nova-2 RNA ligands in filter-binding assays (Fig. 4 C and D). Both Nova-1 and Nova-2 demonstrated high affinity-binding to RNA ligands containing either Nova-1 or Nova-2 selected binding sites. Both proteins bound the Nova-2 selected sequence with approximately equal affinity, but Nova-1 bound its RNA ligand (mSB2) with a slight (2-fold), but consistently higher, affinity than did Nova-2 (Fig. 4 C and D).

## DISCUSSION

**Nova Proteins and Paraneoplastic Disease.** Patients with POMA exhibit clinical symptoms of opsoclonus, myoclonus, and ataxia, suggesting that the initial neurologic disorder involves a lack of motor inhibition in the brainstem and spinal cord pathways (for reviews, see refs. 1, 23, and 27). Expression of the POMA antigen Nova-1 is restricted to the subcortical CNS in a pattern that roughly correlates with these motor symptoms (17, 18). However, a subset of POMA patients also develop encephalopathy and cortical deficits, which cannot easily be explained by dysfunction of Nova-1-expressing neurons (refs. 16 and 22; for review, see ref. 23). Although less well documented than the classic POMA symptoms, the cognitive and emotional features reported in POMA patients range from mild emotional lability to dementia, coma, and death. A review of nine case reports reveals that in 19 adults with POMA, mental status changes were noted in 58%, with progression to stupor or coma in 26% (23).

Analysis of Nova-2 transcripts and protein reveals a widespread expression pattern that includes overlapping expression with Nova-1 in the midbrain, hindbrain, and ventral spinal cord (Fig. 3 and data not shown), as well as strong expression in rostral brain regions, including the cortex and thalamus. The different patterns of Nova expression suggest a model in which progression of subcortical motor system dysfunction to widespread neurologic dysfunction in POMA may correspond to initial immunologic targeting of Nova-1 with progression to targeting of common epitopes shared by Nova-2. We have proposed a similar mechanism for the Hu syndrome, a paraneoplastic syndrome in which progression of focal deficits to multifocal neuronal degeneration may correspond to progressive immunologic targeting of epitopes shared by products of the four Hu genes (10, 15). Such progression of immunologic targeting may be a common feature of autoimmune syndromes involving antigenically related families of disease antigens.

**The Nova Family of Paraneoplastic Disease Antigens.** The Nova-2 cDNA encodes a protein with striking homology to the previously described paraneoplastic antigen Nova-1. In addition, the third KH domain of the predicted Nova-2 protein is identical to the third KH domain of Nova-1, with additional homology in the surrounding sequences; this region has been identified as the antigenic epitope for POMA antisera and also as a functional domain for sequence-specific RNA binding (17,

Nova-1 binding to RNA ligand 87 was observed at  $\approx 65\%$  of the total RNA, while maximal Nova-2 binding was 100%. Similar results were found with the Nova-2 RNA ligand 29 (data not shown). Neither Nova protein demonstrated significant binding to a random RNA ligand isolated from the original RNA selection pool, and an irrelevant T7 epitope-tagged recombinant control protein did not demonstrate significant binding to any of the RNA ligands (calculated  $K_d$  values  $> 1$   $\mu$ M, data not shown).

19). Identification of Nova-2 thus demonstrates the presence of a family of highly related paraneoplastic disease antigens, a finding that is confirmed by recognition of N2FP by 5 of 5 different POMA antisera at the same high titer as Nova-1. Characterization of Nova-2 by immunoprecipitation and Northern blot analysis revealed a 50-kDa, N2Ab-immunoreactive protein and a 3-kb RNA transcript consistent with the Nova-2 sequence reported in Fig. 1B. Moreover, additional protein bands at 75 kDa and RNA transcript(s) at 9 kb were identified. We have shown that the 75-kDa proteins share two distinct epitopes with Nova-2, including the third KH domain (reactivity with POMA antisera) and a 14 amino acid sequence between the second and third KH domains (reactivity with the antipeptide antibody N2Ab). Similarly, the 9-kb RNA transcript hybridizes with a Nova-2 probe in conditions under which crosshybridization to Nova-1 does not occur. Therefore, while the identification of these larger species remains unknown, it is likely that the 9-kb transcript encodes proteins highly related to Nova-2. A Nova-2 probe used in chromosomal mapping studies hybridized with three different loci, none of which were Nova-1 (28), suggesting the possibility of additional Nova genes.

**Sequence-Specific RNA-Binding Proteins in Neuronal RNA Metabolism.** Consistent with the high degree of homology between Nova-1 and Nova-2, the Nova proteins select similar high-affinity RNA ligands in RNA selection experiments and bind individual RNA ligands *in vitro* with similar affinities (Fig. 4; ref. 19). However, in two independent RNA selection experiments, Nova-2 recognized RNA targets with a core consensus sequence (Fig. 4; GAGUCAU) different from a previously identified Nova-1 RNA target [(UCAU(N)<sub>0-2</sub>)<sub>3</sub>; ref. 19]. Moreover, comparison of Nova-1 and Nova-2 binding to [(UCAU(N)<sub>0-2</sub>)<sub>3</sub>] RNA revealed slight but consistent differences in RNA binding (Fig. 4), suggesting that the two Nova proteins may bind overlapping but subtly different sets of RNA ligands. If this is the case, neurons expressing Nova-2 alone may differ subtly in their metabolism of RNA from neurons that express a combination of both proteins (Fig. 3).

Several RNA-binding protein families have been described whose members bind like sequences *in vivo*. HnRNP K and hnRNP E1, two closely related KH-type RNA-binding proteins with homology to the Nova proteins, bind a pyrimidine-rich element in the 15-lipoxygenase mRNA 3' untranslated region and thereby repress mRNA translation (29). Similarly, the KH-type RNA-binding proteins  $\alpha$ CP-1 and  $\alpha$ CP-2 bind  $\alpha$ -globin 3'-untranslated region elements to stabilize the mRNA (30). Finally, the iron-response proteins IRP1 and IRP2 bind to conserved iron response elements in the 5' and 3' untranslated regions of mRNAs involved in regulation of cellular iron levels and function to regulate mRNA stability or translational control (for reviews, see refs. 31 and 32). We have previously shown that Nova-1 protein recognizes the glycine receptor  $\alpha$ 2 pre-mRNA as an *in vivo* RNA target (19). Although the Nova-2 *in vitro* RNA binding data presented here does not necessarily correlate with *in vivo* function, it is possible that the Nova-1 and Nova-2 proteins may share common *in vivo* target RNAs. In such cases, differential regulation of metabolism of RNA targets may be mediated by differential protein-protein interactions. Indeed, preliminary data suggest that Nova-1 and Nova-2 interact to form homo- and hetero-oligomers in solution (Yang and Darnell, unpublished observations), suggesting additional complexity in the regulation of neuronal RNA metabolism by these proteins.

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# A Post-Transcriptional Regulatory Mechanism Restricts Expression of the Paraneoplastic Cerebellar Degeneration Antigen cdr2 to Immune Privileged Tissues

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Paraneoplastic cerebellar degeneration (PCD) is believed to be an autoimmune disorder initiated by the ectopic expression of a neuron-specific protein in breast and ovarian tumors. PCD antisera was used previously to identify several cerebellar degeneration-related (cdr) genes encoding putative PCD antigens. We have found that the cdr2 gene, which encodes a cytoplasmic leucine zipper protein of unknown function, is expressed in PCD-associated tumors, whereas other cdr genes are not; thus, cdr2 encodes the PCD tumor antigen. To determine whether the expression pattern of cdr2 is consistent with its proposed role in PCD, we have isolated the mouse homolog and examined both the mRNA and protein distribution in adult tissues. We have found that cdr2 mRNA is expressed in almost

all tissues, whereas the protein is expressed only in the brain and testis. Within the brain, both the cdr2 mRNA and immunoreactivity are confined primarily to neurons in the cerebellum and brainstem, the regions most affected in PCD. These results suggest first that the tissue-specific expression of cdr2 is regulated at a post-transcriptional level. Moreover, because the brain and testis are considered to be immune-privileged sites, the expression pattern of cdr2 is compatible with the autoimmune model of PCD pathogenesis.

**Key words:** paraneoplastic neurological disease; neuron-specific gene expression; translational regulation; immune privilege; cerebellar degeneration; leucine zipper protein

The paraneoplastic neurological disorders (PNDs) are a rare group of neuronal degenerations that develop as remote effects of systemic malignancies (for review, see Posner and Furneaux, 1990; Darnell, 1996). It is believed that the PNDs are immune-mediated, arising when systemic tumors express proteins (called onconeural antigens) that are normally entirely restricted to their expression to immune-privileged neurons. The serum and CSF of PND patients harbor high titers of anti-neuronal autoantibodies that are also reactive with their tumors (Anderson et al., 1988a; Furneaux et al., 1990; Luque et al., 1991). Characterization of the nature of the disorders and the onconeural antigens has been advanced by the use of PND antisera to clone cDNAs encoding the antigens. Studies of several onconeural antigens, including the breast tumor antigen Nova (Buckanovich et al., 1993, 1995) and the small cell lung cancer antigen Hu (Szabo et al., 1991; Dalmau et al., 1992), have demonstrated that the normal expression of their mRNA and protein is neuron-specific. Taken together with evidence that immunity to PND antigens correlates with effective anti-tumor immunity (Dalmau et al., 1990; Darnell and DeAngelis, 1993; Darnell, 1996), these observations suggest a model for PND in which the expression of onconeural antigens in tumor

cells initiates an appropriate immune response that subsequently develops into autoimmune neurological disease.

Paraneoplastic cerebellar degeneration (PCD) is a PND that develops in patients with breast and ovarian tumors and is characterized by the presence of a specific autoantibody referred to as "anti-Yo" (Anderson et al., 1988b). Immunohistochemical studies with this antibody reveal that it reacts with both PCD-associated tumor cells (Furneaux et al., 1990) and discrete populations of neurons, reacting most strongly with cerebellar Purkinje cell cytoplasm (Cunningham et al., 1986). PCD antisera identify a major antigen reported as either 52 kDa (Tsukamoto et al., 1989; Sakai et al., 1991) or 62 kDa (Cunningham et al., 1986) and a minor antigen of 34 kDa when used in Western blot analysis of Purkinje cell extracts; the larger molecular weight antigen is readily detectable in PCD tumor extracts (Furneaux et al., 1990).

Using PCD antisera, cDNAs encoding three cerebellar degeneration related antigens (cdr1-3) have been identified. cdr1 encodes the 34 kDa protein, the predicted amino acid sequence of which reveals an unusual structure composed of nearly identical hexapeptide repeats making up 91% of the protein (Dropcho et al., 1987). cdr2 was cloned independently from both HeLa cell and human cerebellar cDNA libraries (Fathallah-Shaykh et al., 1991; Sakai et al., 1991). This cDNA encodes a protein with a coiled coil/leucine zipper domain present near the N-terminus. A third cDNA (cdr3) cloned from an HeLa expression library (Fathallah-Shaykh et al., unpublished observations), GenBank accession L02867, shares significant homology (~45% predicted amino acid identity) with cdr2, but has not been investigated further.

It has been suggested that cdr2 may be widely expressed in normal tissues, which is problematic for its proposed role in the pathogenesis of PCD. Widespread expression of cdr2 would also

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be inconsistent with the clinical features of PCD, which are restricted to evidence of anti-tumor immunity and neuronal (primarily cerebellar) dysfunction (Peterson et al., 1992). Early studies using PCD antisera found immunoreactivity with cerebellar Purkinje cells when the antibody was used at limiting dilutions (Jaekle et al., 1985). Subsequent reports have found Purkinje cell immunoreactivity at limiting antibody dilutions, but more widespread reactivity both within and outside the nervous system using high concentrations of antibody (Altermatt et al., 1991; Tomimoto et al., 1993). Furthermore, Sakai et al. (1991) detected the cdr2 mRNA in cerebellum, brainstem, and intestine by reverse transcription PCR (RT-PCR); additional samples were negative but were not controlled for integrity of the RNA.

The present study was undertaken to clarify the expression pattern of the PCD antigen. We have examined three clinical tumors for the expression of cdr genes and found that the cdr2 gene encodes the PCD tumor antigen. We then defined the tissue distribution of the cdr2 mRNA and immunoreactive protein in the adult mouse and have found that expression of the PCD antigen is restricted to the brain and testis, tissues that are recognized as sites of immunological privilege. These results demonstrate that the expression pattern of the PCD antigen is consistent with the proposed autoimmune model of PCD. Interestingly, the cdr2 mRNA displays a wider distribution than the protein, indicating that expression of the cdr2 antigen is regulated at a post-transcriptional level.

## MATERIALS AND METHODS

**Tumor RNA extraction and RT-PCR.** Frozen samples of ovarian tumors removed from patients with Yo-positive PCD were obtained from the Memorial Sloan Kettering Cancer Center. Fragments (100 mg) were used for purification of either total or poly(A<sup>+</sup>) RNA. Total RNA was prepared by the method of Chomczynski and Sacchi (1987), and the samples were subsequently treated with RQ1 RNase-free DNase (Promega, Madison, WI) before RT-PCR analysis. For the purification of poly(A<sup>+</sup>) RNA, the section was homogenized by sonication in 400  $\mu$ l extraction buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 8.0, 1% dithiothreitol, 0.5% lauryl sarcosinate), and 800  $\mu$ l of binding buffer (0.1 M Tris-HCl, pH 8.0, 0.4 M LiCl, 20 mM EDTA) was added and the lysate clarified by centrifugation at 18,000  $\times$  g for 5 min. Magnetic Dynabeads Oligo (dT)<sub>25</sub> (Dyna, Great Neck, NY) were prepared by washing 300  $\mu$ l of beads once with 200  $\mu$ l binding buffer. The lysate supernatant was added to the Dynabeads, mixed, and incubated at room temperature for 3–5 min. A magnet was used to immobilize the beads, the supernatant was removed, and the beads were washed three times with 0.5 ml wash buffer (10 mM Tris-HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA). Poly(A<sup>+</sup>) RNA was then eluted in 20  $\mu$ l 2 mM EDTA, pH 8.0, at 65°C for 2 min.

For RT-PCR reactions, 2  $\mu$ l tumor poly(A<sup>+</sup>) RNA, 1–2  $\mu$ g total tumor RNA, or 20 ng poly(A<sup>+</sup>) RNA from normal tissues (CLONTECH Laboratories, Palo Alto, CA) was denatured at 70°C for 10 min and placed on ice. The RNA was reverse-transcribed using random hexanucleotide primers (Boehringer Mannheim, Indianapolis, IN) and Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) at 42°C for 50 min and the reaction stopped by incubation at 95°C for 5 min. One-tenth of the first strand cDNA sample was used as template for a PCR reaction using AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT) and the following forward and reverse oligonucleotide primers corresponding to cdr2: 5'-TGAATGGAGTTGAGA AGCTGGTG-3' and 5'-GAGATGCCCTCTGTTTCACAG-3'; and cdr3: 5'-CATTGAGCGCCTCCAGGCT-3' and 5'-AGTCCTTGAGG CAGGGGAA-3'. The product was amplified for 35 cycles (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min). PCR products were labeled by addition of trace amounts of [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham Life Science, Arlington Heights, IL) to the reaction mixtures and loaded on a 10% nondenaturing acrylamide gel and visualized by autoradiography.

**cDNA library screening and sequencing.** Adult mouse brain and spleen cDNA libraries (Stratagene, La Jolla, CA) were plated at a density of  $1 \times 10^5$  pfu per 135 mm dish. Plaques were lifted onto nitrocellulose filters for hybridization with <sup>32</sup>P-labeled probes (Sambrook et al., 1989). The mouse brain library ( $1.2 \times 10^6$  pfu) was screened at low stringency with a probe

corresponding to bp 1–872 of the human cdr2 cDNA (Fathallah-Shaykh et al., 1991). This screen resulted in the isolation of a single 2.4 kb clone. The mouse spleen library ( $6 \times 10^5$  pfu) was screened with a cDNA probe corresponding to bp 153–720 of the mouse brain cdr2 cDNA (see Fig. 2). This screen resulted in the isolation of three overlapping clones, one comprising the full coding regions and untranslated regions (UTR). The cDNA clones were sequenced by the dideoxy method of Sanger et al. (1977) using Sequenase 2.0 (United States Biochemical, Cleveland, OH), and sequence data were analyzed using the MacVector software package (International Biotechnologies, New Haven, CT).

**Northern blot analysis.** Adult ICR (Charles River) mouse organs were dissected, and total RNA was prepared using the TRIZOL Reagent (Life Technologies) and the protocol recommended by the manufacturer. Total RNA (30  $\mu$ g) was resolved on an agarose/50% glyoxal gel (Sambrook et al., 1989) and transferred to Biotodyne B nylon membrane (Pall, Glen Cove, NY). The RNA was UV cross-linked to the membrane, and the blot was prehybridized in 6 $\times$  SSC, 0.5% SDS, 5 $\times$  Denhardt's solution, 0.05% sodium pyrophosphate, 100  $\mu$ g/ml denatured herring sperm DNA for 30 min at room temperature. cDNAs corresponding to a 580 bp fragment of the mouse cdr2 3'-UTR and the full-length GAPDH coding region were labeled with [<sup>32</sup>P]dCTP using the Prime-It Kit (Stratagene). The RNA blot was hybridized with  $1 \times 10^6$  cpm/ml labeled probe in 6 $\times$  SSC, 1 $\times$  Denhardt's solution, 0.05% sodium pyrophosphate, 100  $\mu$ g/ml yeast tRNA for 16 hr at 65°C. The membrane was then washed twice in 2 $\times$  SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 65°C, and twice in 1 $\times$  SSC, 0.1% SDS, 0.1% sodium pyrophosphate at room temperature, and hybridization was visualized by autoradiography.

**Antibody affinity purification and Western blot analysis.** The region coding for amino acids 16–192 of human cdr2 was fused in frame to glutathione-S-transferase (GST) in the GSTag vector (the gift of David Ron, New York University). Bacteria transformed with the GSTag-cdr2 plasmid were grown to an OD<sub>600</sub> of 0.5 and 1 mM isopropyl thiogalactoside added for an additional 3 hr. Cells were harvested and resuspended in ice-cold PBS and lysed by sonication, and the lysate cleared by centrifugation. The cleared lysate was incubated with glutathione Sepharose (Pharmacia Biotech, Piscataway, NJ) and washed in PBS, and fusion protein eluted with 10 mM reduced glutathione. Purity and immunoreactivity of the GST-cdr2 fusion protein were verified by SDS-PAGE, Coomassie blue staining, and Western blot analysis.

For affinity purification of PCD antisera, Immobilon membrane (Millipore, Bedford, MA) was wetted with methanol and rinsed well with ddH<sub>2</sub>O. GST-cdr2 fusion protein (20–30  $\mu$ g) was spotted on a 0.5  $\times$  3 cm strip of membrane and blocked for 60 min in 25 mM Tris-HCl, pH 8.0, 20 mM Na<sub>2</sub>CO<sub>3</sub>, 150 mM NaCl, 5% nonfat dry milk. The strip was washed with PBS/0.02% sodium azide and incubated with 1 ml of PCD antiserum for 2 hr at 4°C. The strip was then washed four times with 25 mM Tris-HCl, pH 8.0, 20 mM Na<sub>2</sub>CO<sub>3</sub>, 150 mM NaCl, 0.1% Triton X-100, two times with 25 mM Tris-HCl, pH 8.0, 20 mM Na<sub>2</sub>CO<sub>3</sub>, 150 mM NaCl, 2 mM EDTA, and antibody was eluted with 1 ml 0.2 M glycine, pH 3.0. The elution was repeated, and the pooled eluates were neutralized with 0.5N NaOH to a final pH of 7.5–8.0. Ultrafiltration in a Centricon-10 unit (Amicon, Beverly, MA) was used to remove the glycine from the affinity-purified antibody.

For Western blot analysis, the indicated tissues were dissected from adult ICR mice and homogenized in PBS, 2 $\times$  SDS sample buffer added, and the samples boiled. Frozen sections of PCD ovarian tumors were pulverized with a mortar and pestle under liquid nitrogen and homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM potassium fluoride, 1% NP-40, 5 mM EDTA). Total protein (45  $\mu$ g) from each tissue extract was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated with affinity-purified PCD antibody diluted to 1:50 or native PCD antisera diluted to 1:400, washed, and incubated with anti-human IgG conjugated to horseradish peroxidase (Amersham) at a 1:5000 dilution. Reactive proteins were detected using the ECL kit (Amersham) according to the manufacturer's instructions and stripped of antibody according to the ECL protocol.

**Two-dimensional (2-D) gel electrophoresis.** Cerebellum and testis from Sm/Ckc mice were homogenized in 2-D lysis buffer [9.5 M urea, 2% NP-40, 5%  $\beta$ -mercaptoethanol, 2% Biolyte ampholytes (BioRad Labs, Hercules, CA) consisting of 75% 3/5 range and 25% 3/10 range Biolytes]. The lysate was clarified by centrifugation at 2100  $\times$  g for 5 min, and protein concentrations were adjusted with 2-D lysis buffer. Isoelectric focusing (IEF) gels were performed essentially by the method of O'Farrell (1975). IEF slab gels [0.75 mm; 9.2 M urea, 4% acrylamide (ReadySol IEF, Pharmacia Biotech), 2% NP-40, and 5% Biolyte am-



pholytes] were loaded with 40  $\mu$ g of total protein per lane, and the samples were covered with sample overlay buffer (7% urea, 2.5% ampholytes, 5%  $\beta$ -mercaptoethanol). The gels were run using 0.01 M  $H_2PO_4$  and 0.02 M NaOH buffers, as described by O'Farrell (1975) at 4 W constant power. Voltage was limited to 700 V, and gels were run for 1800 V/hr. Lanes containing the samples were cut from the gel, equilibrated with 1 $\times$  SDS sample buffer for 5 min, and loaded horizontally onto a 1 mm 9% SDS-PAGE gel with a 3% stacking gel. A single well was loaded with 40  $\mu$ g protein extract in SDS sample buffer for one-dimensional analysis. Gels were transferred to nitrocellulose and probed with PCD antisera.

**In situ hybridization.** The protocol used was essentially the same as that described by Newman et al. (1995). Adult ICR mouse tissues were embedded and frozen in O.C.T. compound (Miles, Elkhart, IN). Sections (10  $\mu$ m) were cut using a cryostat and applied to Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). A plasmid containing the same region of the 3'-UTR of the mouse cdr2 gene as that used for the Northern blot was linearized and both sense and antisense riboprobes transcribed using T7 RNA polymerase (Stratagene) and [ $^{33}P$ ]UTP (Dupont NEN, Boston, MA). Probe was purified on a Sepharose G50 column and hybridized to tissue sections at 50°C for 36–48 hr.

**Immunohistochemistry.** For the human tissues, IgG from PCD and normal human sera was isolated and biotinylated, as described previously (Furneaux et al., 1990). Paraffin-embedded sections of ovarian tumor and cerebellum from PCD patients were reacted with the biotinylated antibodies, as described by Verschuuren et al. (1996).

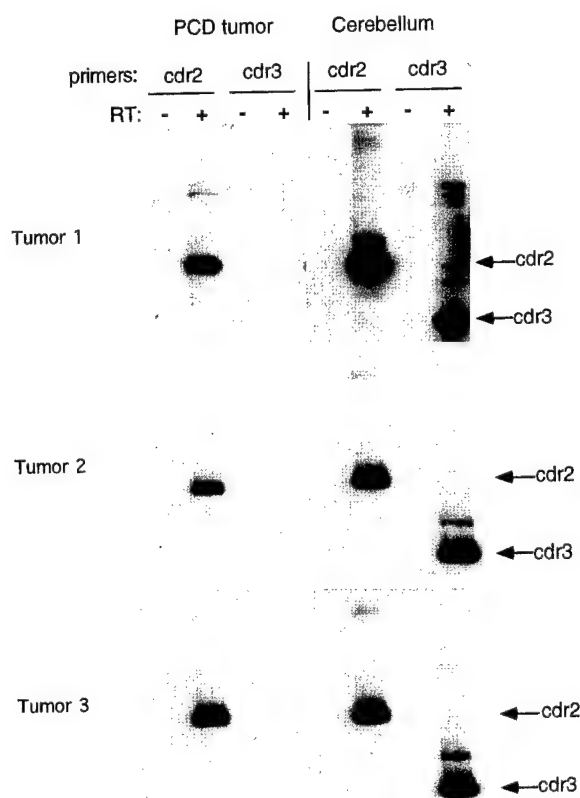
For the mouse tissues, whole organs were dissected from adult ICR mice and tissues were embedded and frozen in O.C.T. compound. Sections (10  $\mu$ m) were fixed in methanol/0.3%  $H_2O_2$  at room temperature for 30–60 min to quench endogenous peroxidase activity, washed in PBS, and blocked with PBS/2% normal goat serum (NGS) at room temperature for 1 hr. Sections were incubated with primary antibody diluted in PBS/2% NGS at 4°C overnight, washed in PBS, and incubated with biotinylated anti-human IgG (Vector Laboratories, Burlingame, CA) diluted 1:5000 in PBS/2% NGS at room temperature for 1–2 hr. The signal was enhanced by addition of an avidin–biotin complex (Vectastain Elite Kit, Vector Laboratories) and visualized with diaminobenzidine in the presence of  $H_2O_2$ .

## RESULTS

### Detection of cdr2 message in PCD-associated ovarian tumors

Previous studies demonstrated that PCD antisera recognized a protein of ~62 kDa in all PCD tumor samples examined but detected the 34 kDa species (cdr1) in only one (Furneaux et al., 1990), suggesting that cdr2 might encode the PCD tumor antigen. However, the antigenic epitope in cdr2 localizes to the leucine zipper domain of the protein (Sakai et al., 1993), which is nearly identical in sequence to the leucine zipper of cdr3, and both cDNAs were cloned using PCD antisera. To address which of these two genes encodes the *in vivo* PCD tumor antigen, we assayed PCD tumors for the presence of cdr gene transcripts by RT-PCR. Single-strand cDNA was synthesized from poly(A<sup>+</sup>) or total RNA isolated from three ovarian tumors obtained from PCD patients and amplified using gene-specific primers corresponding to cdr2 or cdr3 (Fig. 1). Although both cdr transcripts could be detected in the cerebellum, only cdr2 mRNA was found in the PCD ovarian tumors. In addition, cdr1 mRNA was detected in cerebellum but could not be detected in the PCD tumors (data not shown).

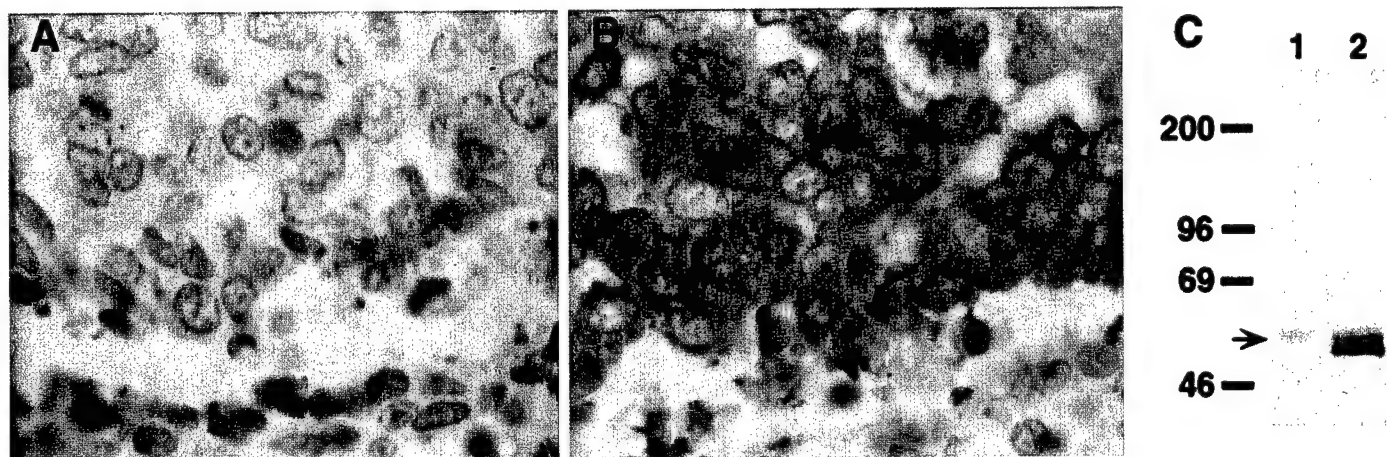
To confirm that the cdr2-positive PCD tumors we assayed came from typical PCD patients, we examined tumor tissue for immunoreactivity with PCD antisera. Figure 2 demonstrates that tumor tissue from one patient (tumor 2) was immunoreactive with PCD antisera (Fig. 2B) but not control antisera (Fig. 2A); similar results were found with tumors 1 and 2 (data not shown). In addition, we assayed tissue from tumor 3 for PCD reactivity by Western blot analysis. Figure 2C demonstrates that PCD antisera recognized a protein that comigrates with the PCD antigen rec-



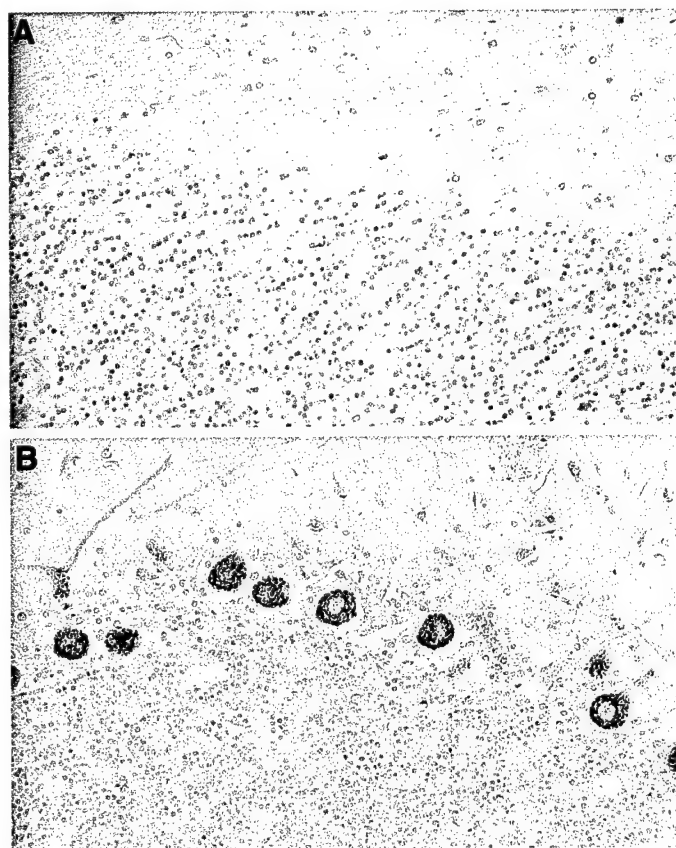
**Figure 1.** RT-PCR analysis of PCD-associated ovarian tumors. Total or poly(A<sup>+</sup>) RNA purified from tumor tissue or human cerebellar poly(A<sup>+</sup>) RNA was used as templates for the RT. Gene-specific primers corresponding to the coding region of either cdr2 or cdr3 were used for PCR amplification of the first strand cDNA. Reactions were performed both in the presence and absence of RT to control for DNA contamination. A  $\beta$ -actin primer pair was also used as a control for RNA integrity (data not shown). Although the transcripts of the expected size for both cdr2 and cdr3 were detectable in human cerebellum, only the cdr2 transcript was detected in the PCD tumors.

ognized in human Purkinje extracts. Finally, we examined cerebellar tissue obtained from the autopsy of patient 3. Immunohistochemical analysis of cerebellar tissue using PCD antisera revealed the complete absence of immunoreactivity and Purkinje neurons in PCD cerebellum (Fig. 3A) but showed a characteristic staining pattern in Purkinje neurons of control cerebellum (Fig. 3B). We conclude from the RT-PCR and protein studies that the PCD tumor antigen is the cdr2 gene product.

To facilitate the study of cdr2 expression, we used a human cdr2 cDNA clone (Fathallah-Shaykh et al., 1991) to isolate a 2.4 kb cDNA encoding cdr2 from an adult mouse brain library (Fig. 4). Because the initiating methionine of the human cDNA has not been defined (Fathallah-Shaykh et al., 1991; Sakai et al., 1991), we compared the degree of nucleic acid homology between the murine and human cdr2 sequences. The sequence homology decreases dramatically immediately upstream of a methionine codon at position 154, and there is an in-frame stop codon at position 88, indicating that this ATG is the initiation codon. The full-length murine cdr2 open reading frame encodes a protein of predicted molecular weight 52 kDa, and its amino acid sequence is 87% identical to its human homolog (Fig. 4). Within the region of the antigenic epitope (the leucine zipper domain), the mouse and human proteins are identical.



**Figure 2.** Above. Immunoreactivity of PCD ovarian tumors with PCD antisera. Serial sections of a paraffin-embedded PCD ovarian tumor (tumor 2 from Fig. 1) stained with either biotinylated normal human serum (*A*) or biotinylated PCD antisera (*B*). PCD antisera displays a characteristic cytoplasmic reactivity in the tumor tissue and not in the surrounding connective tissue seen in the bottom of the photomicrograph. *C*, Detection of the PCD ovarian tumor antigen by Western blot. PCD antisera was immunoreactive with a protein of  $M_r$  56 kDa (arrowhead) present in both human Purkinje (*lane 1*) and PCD tumor (*lane 2*) protein extracts. The lower reactive band in the tumor extract is IgG, determined by probing the same blot with the anti-human IgG secondary antibody alone (data not shown).



**Figure 3.** Left. Immunohistochemical analysis of normal or PCD cerebellar sections. *A*, Paraffin section of the cerebellum of a PCD patient (corresponding to tumor 3 in Fig. 1) showing complete lack of reactivity with biotinylated PCD antiserum and the absence of Purkinje neurons. *B*, Paraffin section of the cerebellum of a neurologically normal patient demonstrating reactivity of Purkinje cell cytoplasm with PCD antiserum.

### cdr2 mRNA is expressed widely in adult mouse tissues, but the protein is restricted to the brain and testis

To determine whether cdr2 gene expression was limited to the nervous system, we performed Northern blot analysis using a cdr2-specific cDNA probe. A single cdr2 transcript of 2.8 kb was detected in eight of nine tissues examined; it was most abundant in testis and spleen and was not detectable in liver (Fig. 5*A*). This result was confirmed by hybridization with a probe from a different region of the cdr2 gene (data not shown) and by RT-PCR analysis of mouse cerebellum, spleen, heart, and testis RNA (Fig. 5*B* and data not shown). The cdr2 primer pair used for PCR amplification flanks the region encoding the PCD epitope, suggesting that this region of the cdr2 transcript is the same in each tissue.

To investigate the expression pattern of the PCD antigen, Western blot analysis using affinity-purified PCD antisera was performed on the same battery of adult mouse tissues used for Northern blot analysis. Interestingly, a single band of  $M_r$  = 56 kDa was detected in cerebellum and testis but not in other tissues (Fig. 6*A*). On a longer exposure, a faint band was also detected in the cerebral cortex (data not shown). The blot was stripped of antibody and reprobed with a monoclonal antibody to  $\beta$ -tubulin (Fig. 6*A*), demonstrating that equivalent amounts of protein were loaded in each lane. To confirm the identity of the immunoreactive species in brain and testis, we performed 2-D gel electrophoretic analysis. Figure 6*B* shows that the protein recognized by PCD antisera in cerebellum (*top panel*) and testis (*bottom panel*) exactly comigrate by both their molecular weights and isoelectric points (pI). The protein(s) runs

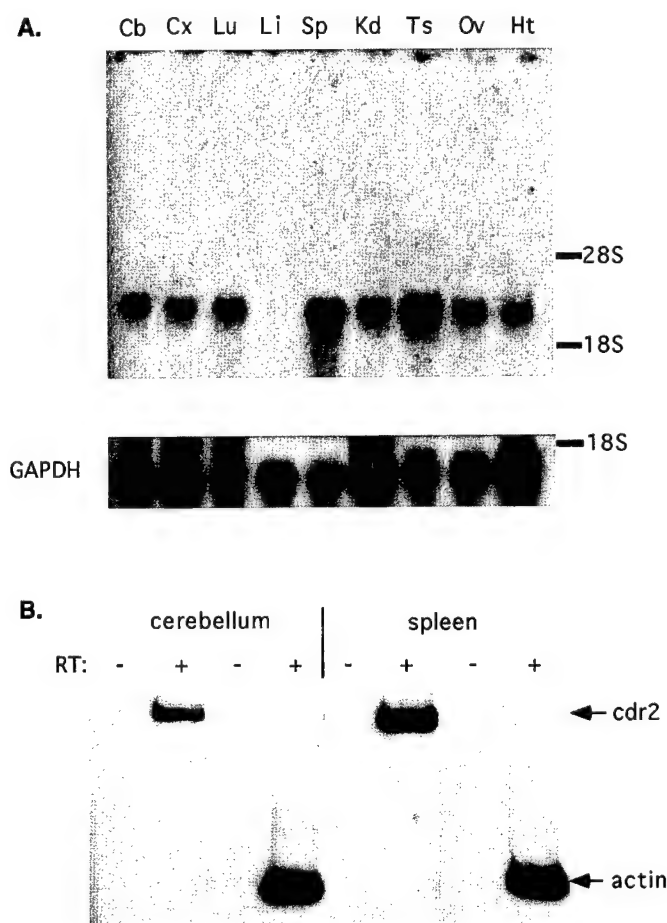
	ttgggcccggcagcagcgctgaggagccggctcccggcgccacccggggcgcgctcgctagggcgccggcct	72
	ttgggcccggcgga <u>tag</u> cgcgagagctgctgcgggacctgcgtgtccgcccagggtggctctggctgag	144
	ggcggcgacatgctggcagataacctagtggaggagtgcgagatggaggatgagccgtggtagcaccacgg	216
1	M L A <u>D</u> N L V E E F E M E D E P W Y D H R	
	gacctccagcaagatctccagcttgctgctgaacttgaaagacgctactggatcggaacacagagttggaa	288
22	D L Q Q D L Q L A A E L G K T L L D R N T E L E	
	gattctcttcagcagatgtacacaaccaatcaggagcagttacaggaaatcgagtacctgaccaagcaggtg	360
46	D S L Q Q M Y T T N Q E Q L Q E I E Y L T K Q V	
	gagcttctacggcaaatgaatgagcagcatgcgaaagtttatgagcagtttagatgtcacagcaagagaactg	432
70	E L L R Q M N E Q H A K V Y E Q L D V T A R E L	
	gaagaaaccaaccagaagctagtgtgtagagcaaggcctcacagcagaaaatcctcagcctgacagaaaca	504
94	E E T N Q K L V A <u>E</u> S K A S Q Q K I L S L T E T	
	attgaatgcctgcaaaccaacattgatcacctgcagagccaagtggaggagctgaagtcttccagccaagga	576
118	I E C L Q T N I D H L Q S Q V E E L K S S <u>S</u> Q G	
	agaggagcagaagcagtgatgaccaggagaaccagcaccagcttctcctgtctgaaagagctgtatgac	648
142	R <u>G</u> R Q K <u>A</u> C D Q E K P A P S F <u>S</u> C L K E L Y D	
	ctccgccaacactttgtgtatgaccacgtgttcgctgagaagatcacttcttgcagagccaacagagccct	720
166	L R Q H F V Y D H V F A E K I T S L Q <u>S</u> Q Q S P	
	gatgaagaagaaaatgagcacctgaaaaaggcagtgacgatgttgaggccagcctaagtctagagaggaag	792
190	D E E E N E H L K K <u>A</u> V T M L Q A Q L S L E R K	
	aagcagtgagcgtggaggcagagtataaagtgggtgctgaaggagaacagtgagctggagcagcagctgggg	864
214	K R V <u>S</u> <u>V</u> E <u>A</u> E Y <u>K</u> <u>V</u> V L K E N S E L E Q Q L G	
	gccacagatgcctaccgagccccggccctggagttggaggcagaggtggctgagatgcggcagatgtgcag	936
238	A T <u>D</u> A Y R A R A L E L A E V A E M R Q M L Q	
	gcagtagctatcctttgtgaatggtgtggagaagctggtgcccactctctgttttcttcttcaaggagcct	1008
262	<u>A</u> E H P F V N G V E K L V P D S L F V P F K E P	
	agccagagtctgctggaggaaatgttctggtgctccagaagcacctagaaaaccactcaaacgaagcagc	1080
286	S Q S L L E E M F L <u>A</u> <u>A</u> P E <u>A</u> P R K P L K R S S	
	agtgcagcgcgctcagcagtttggcaggggatgacatcgtagaggaccatgaggacacttgcacagcaggt	1152
310	S E T <u>A</u> L S S L A G <u>D</u> D I V K <u>D</u> H E <u>D</u> T C I R R	
	gctaaggctgtgaagcagaggggctccttctgcatgaagtggacactcagtagcagctgacctgaaagt	1224
334	A K A V K Q R G I S L L H E V D T Q Y S A L K V	
	aagtatgaagagctactgaagaagtgccacgaggagcaggactcactgtcacacaaggctgtgcagacctct	1296
358	K Y E E L L K K C <u>H</u> E E Q D S L S H K A <u>V</u> Q T <u>S</u>	
	agactactgactagggacctgacaggactggtcacgcagctctgaggcggggccagtggtgggaaccacc	1368
382	R L L T R D L T <u>G</u> L V T Q S E <u>A</u> <u>G</u> A S G W E P T	
	cctgtaagcccagagtccatcagttccccaccactacacctccagaatacaaaagcactatttaaggagata	1440
406	<u>P</u> V <u>S</u> P E <u>S</u> <u>I</u> S S P T T T P P E Y K A L F K E I	
	tttagttgcatcaagaaaacaaagcaggaatagatgaacagagaacaaaataacccttctctcctcttac	1512
430	F S C I K K T K Q E I D E Q R T K Y <u>P</u> S L S S <u>Y</u>	
	tcgtactgagactccagttgtaactgcttgttttcgctcacttgccctccngttcagacatgtaagtgcagt	1584
454	S Y *	
	ctccaaagcgttgacgttgctaatgacacctgcctcggtgcttctgcttagttggcaaaagcaggaagaggt	1656
	gacagagaatgttgactccaaggtccctaggaatctcacgatacactggccgctggttggtacactgattt	1728
	ccagaaaagccccagaagcagtagaggagagccatgtacttgggagtaggctgaagtacctaacagtgtta	1800
	gggtaaagaacagaattggaacagaactaaggctgttgccttcatagcatctcagactacttggaaactcaa	1872
	ggactgcctaactaaagtactttcacaaactaacattcagaaggatgttgagaaagcaagatctaactcg	1944
	aaatggatgacattaacaagctccttaagccccctgaagttttattgcatctgtagcacttgaatgctgact	2016
	gacaggcagatcctgatataatccaaattcgaattaataccatttgaaatgnaagctcttnttaacgcctgtt	2088
	tgtatgcccagcattcagctgttttactcaaagctaggcatgtcatagtaattgtcttttcagatggaaact	2160
	agagaactctcctaggtgtgatctgcactggccacttggcaggcacaagctgcttcttccctcaggtctt	2232
	aacccttccagacctgcagcagattcctgtcagactcccttacacagtattgatcttctgactgtggaaacca	2304
	accttctctcactttcacacctttaattaaatctatttaagagaacctggttctgattgtgtgtatat	2376
	ttttctaaaggctgataaaagctatctatgaatgtgaaaaaaaaaaaaaaaaaaaaa	2443

**Figure 4.** Nucleotide and predicted amino acid sequence of the adult mouse brain cdr2 cDNA. Amino acids are numbered on the left and the nucleotides on the right. The in-frame stop codon upstream of the presumptive initiating methionine and a polyadenylation signal are underlined. The translational stop codon is indicated with an *asterisk*. The 455 amino acid protein of predicted  $M_r = 52$  kDa is 87% identical to the human sequence. The amino acids that are not conserved are underlined.

as a broad band with a pI of 6.1–6.4, consistent with previous 2-D gel analysis of human Purkinje cell lysate (Cunningham et al., 1986). This migration pattern in the dimension of isoelectric focusing could be attributable to post-translational or chemical modifications and may also explain the difference between the observed pI and the predicted pI of 4.76.

There are several potential explanations for the discrepancy between the tissue distribution of the cdr2 message and the protein detected by the PCD antisera. In tissues other than brain and testis, the PCD antigen may not be recognized by the Yo antibody as a result of alternative processing of the primary transcript, differential post-translational modification of the pro-





**Figure 5.** Expression of the cdr2 mRNA in adult mouse tissues. *A*, A Northern blot of total RNA prepared from the indicated tissues was hybridized with a  $^{32}$ P-labeled cDNA probe made from the 3'-UTR of mouse cdr2 (*top panel*). The *bottom panel* shows hybridization of a GAPDH probe to the same blot as a control for loading of RNA. The mouse tissues used in the analysis were cerebellum (*Cb*), cerebral cortex (*Cx*), heart (*Ht*), lung (*Lu*), liver (*Li*), kidney (*Kd*), spleen (*Sp*), ovary (*Ov*), and testis (*Ts*). The relative positions of 28S (5.1 kb) and 18S (2.0 kb) rRNA are shown. A single cdr2 transcript of 2.8 kb was detected in all tissues tested, with the exception of the liver. *B*, RT-PCR analysis of cdr2 expression in cerebellum versus spleen RNA was performed, as described in Figure 1, using primers flanking the PCD epitope or  $\beta$ -actin primers. The cdr2 transcript was also detected in heart and testis by this assay (data not shown).

tein, or regulation at the level of translation. To test for the first of these possibilities, we cloned cdr2 cDNA from spleen. Mouse spleen was chosen, because it represents a tissue in which the cdr2 mRNA was abundant, whereas the protein was undetectable. Two overlapping clones, one nearly full-length, were isolated and found to be identical to the brain cDNA sequence throughout the coding region and UTR. These results suggest that the apparent differences in detectable cdr2 protein in brain and spleen cannot be accounted for by alternatively processed cdr2 mRNAs.

To address the possibility that the detection of cdr2 may be affected by tissue-specific post-translational modifications or protein stability, we transfected a non-neuronal cell line with an expression vector containing the full cdr2 open reading frame without UTR sequences. The transfected cdr2 plasmid yielded abundant immunoreactive protein in NIH3T3 cells, suggesting that the protein was stable and the epitope was not masked in this fibroblast cell line (data not shown).

### cdr2 expression is also regulated at the level of transcription

To extend our cdr2 expression data, the tissue distribution of cdr2 mRNA and protein was compared by *in situ* hybridization and immunohistochemistry. A specific *in situ* hybridization probe was generated from the mouse cdr2 3'-UTR, and the expression in mouse tissues was compared with the pattern of immunoreactivity seen with affinity-purified or native PCD serum. Both sagittal and coronal sections of adult mouse brain showed a pattern of cdr2 mRNA expression that corresponded precisely to the pattern of immunoreactivity. Abundant cdr2 mRNA and PCD antigen was detected specifically in the cerebellar Purkinje cells, in neurons of the deep cerebellar nuclei, and in brainstem (Fig. 7*A,B*). cdr2 mRNA and protein were absent from hippocampus, basal ganglia, and neocortex, with the exception of scattered immunoreactive cortical neurons (data not shown).

Outside the nervous system, there was no correlation between cdr2 *in situ* hybridization and immunohistochemistry except in testis. In sections of adult spleen, the cdr2 mRNA was readily detected, where it was found to be restricted to the splenic cortex (an area rich in lymphocytes) but absent from the red pulp (Fig. 7*C*). In contrast, there was no detectable cdr2 immunoreactivity in either the splenic cortex or the pulp (Fig. 7*D*). Similarly, no immunoreactivity could be detected in any other non-neuronal tissue examined except testis. Immunohistochemical staining of testis revealed that cdr2 cytoplasmic reactivity was restricted to the outermost cell layer of the seminiferous tubules (Fig. 7*F*). By their relative position in the tubules and by morphological criteria, these cells appear to be spermatogonia, the least-differentiated type in the germ cell lineage. *In situ* hybridization of testis revealed that cdr2 mRNA is abundantly expressed in spermatogonia and could be detected to a lesser degree in early differentiating spermatocytes (Fig. 7*E*). Taken together, these data demonstrate an uncoupling of cdr2 mRNA and protein expression, suggesting that a post-transcriptional mechanism restricts cdr2 protein expression to spermatogonia and a subset of neurons.

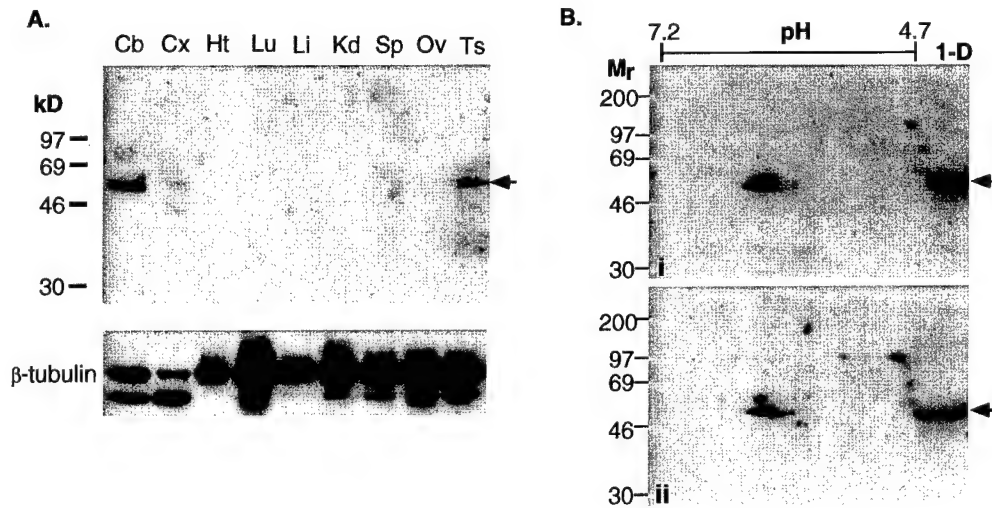
## DISCUSSION

### The onconeural antigen cdr2

Three genes encoding putative PCD antigens have been identified by expression library screening with PCD antisera (Dropcho et al., 1987; Fathallah-Shaykh et al., 1991; Sakai et al., 1991), but which of these encode proteins that might be relevant to PCD *in vivo* has been uncertain. Western blot analysis of PCD tumor tissue probed with PCD antisera previously identified immunoreactive species that might correspond in size to either the cdr2 or cdr3 gene products (Furneaux et al., 1990). Moreover, the cdr2 and cdr3 genes encode proteins that share a common epitope (the leucine zipper domain). We have performed RT-PCR analysis of three PCD-associated ovarian tumors and found that in each of these tumors, the only PCD-related gene to be expressed is cdr2, and we conclude that the cdr2 protein is the PCD tumor antigen.

Based on RNA analysis and immunohistochemical studies (Sakai et al., 1991; Tomimoto et al., 1993), it had been thought previously that the PCD antigen cdr2 might be expressed outside the nervous system, generating uncertainty regarding its role as an onconeural antigen. We have demonstrated that the expression of the PCD antigen is normally restricted to neurons and testis, sites exhibiting the characteristics of immune privilege. Two-dimensional gel analysis confirms that the immunoreactive species evident on Western blot analysis is the same protein in both

**Figure 6.** Detection of the PCD antigen in adult mouse tissues. **A**, Affinity-purified PCD antisera was used to probe a Western blot of the indicated protein extracts (*top panel*, abbreviated as in Fig. 3). The blot was stripped of antibody and reprobed with a monoclonal antibody to  $\beta$ -tubulin as a protein-loading and transfer control (*bottom panel*); the lower band is mouse Ig heavy chain. The cdr2 affinity-purified antibody recognizes a  $M_r$  56 kDa antigen only in brain and testis. **B**, Two-dimensional gel electrophoresis of protein extracts from cerebellum (*i*) and testis (*ii*). Proteins were resolved by their isoelectric points in the horizontal direction (the direction and end points of the pH gradient are shown above) and by their molecular weights in the vertical direction. The major species detected by PCD antisera in cerebellum and testis comigrate in both dimensions.



tissues. This expression pattern is consistent with the proposed role of cdr2 as an onconeural antigen.

The expression of previously characterized onconeural antigens and some autoimmune antigens has been found to be very tightly restricted to neurons. The Nova and Hu onconeural antigens are RNA-binding proteins expressed exclusively in neurons both early in embryogenesis and in adults (Szabo et al., 1991; Dalmau et al., 1992; Buckanovich et al., 1993, 1995). Similarly, the autoimmune cerebellar degeneration antigen  $\beta$ -NAP is a neuron-specific vesicle coat protein (Newman et al., 1995), and the stiff-man syndrome antigens GAD (Solimena et al., 1988) and amphiphysin (DeCamilli et al., 1993) are nerve terminal vesicle-associated proteins. Our findings are thus concordant with the strict regulation of onconeural antigen expression but indicate that their expression may extend to immune-privileged cells outside the nervous system.

The phenomenon of immune privilege, traditionally described as the prolonged survival of allogeneic or xenogeneic grafts, has been studied most extensively in the brain, eye, and testis (Streilein, 1993). Immune privilege in the nervous system has both a physical and a molecular component, defined by the blood–brain barrier and the lack of detectable MHC class I or II antigens, respectively (Bradbury, 1984; Pollack and Lund, 1990). There are also active mechanisms by which immune-privileged tissues evade immune surveillance. For example, cells in the anterior chamber of the eye and the testis express fas ligand as a means of inducing apoptosis of autoreactive immune cells (Bellgrau et al., 1995; for comments, see Griffith et al., 1995). Although these studies have examined the immune response to foreign antigens expressed within immune-privileged tissues, they imply that proteins normally restricted in their expression to such sites may be highly immunogenic when ectopically expressed.

In PND, it is believed that sequestration of onconeural antigens from immune surveillance in the brain results in lack of immune tolerance to these proteins when they are ectopically expressed in tumor cells. We have shown that cdr2 is the only cdr gene expressed in ovarian tumors from PCD patients, and thus appears to be the inciting onconeural antigen. Ectopic expression of cdr2 is associated with a robust immune response to the antigen. The presence of a specific high-titer autoantibody and limited tumor growth in PCD patients provide clinical evidence for an active anti-tumor immune response (Anderson et al., 1988b; Peterson et

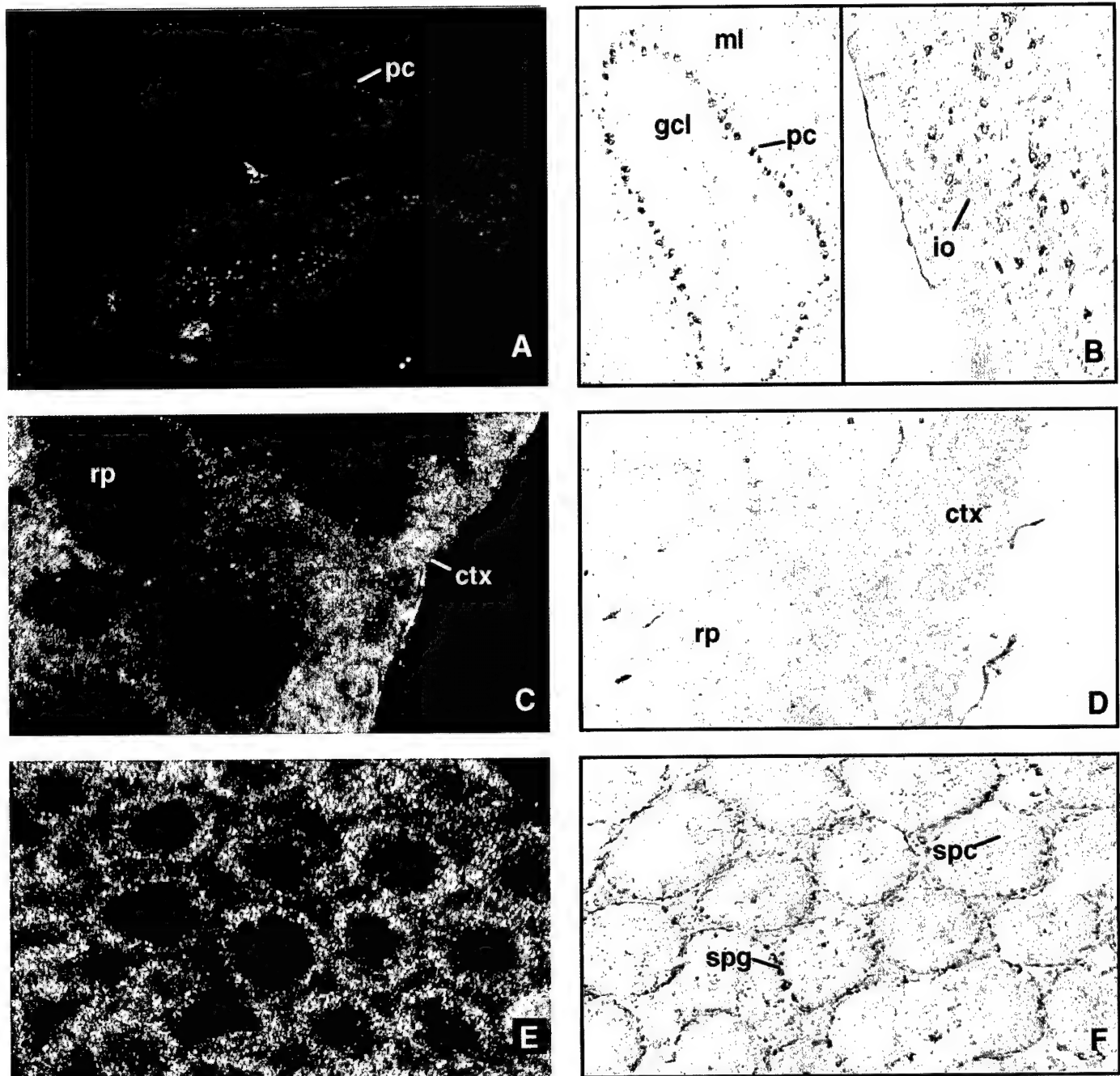
al., 1992) and suggest that the cdr2 protein may act as a *bona fide* tumor antigen.

It remains uncertain how a systemic immune response to ectopically expressed cdr2 protein becomes competent to recognize the antigen within the brain. However, it does appear that the autoimmune response within the nervous system in PCD is likely to be directed against cdr2. Pathological examination of PCD brains reveals degeneration of the same neurons in which cdr2 is expressed, most prominently Purkinje neurons of the cerebellum (Fig. 3) (Peterson et al., 1992; Verschuuren et al., 1996). Taken together, these observations suggest that autoimmunity to cdr2 in PCD proceeds in two steps. First, a naive immune system is naturally competent to recognize cdr2 in PCD tumors. A second unidentified event, perhaps involving cytokines or a change in the nature of the cellular immune response, allows the immune privilege of the brain to be breached, culminating in autoimmune neurological disease.

### Regulation of the cdr2 antigen at a post-transcriptional level

We have clarified the nature of PCD by definitively identifying cdr2 as the neuronal gene that is ectopically expressed in PCD tumors. Given the significance of such onconeural genes to tumor biology and neurobiology (for review, see Darnell, 1996), this observation focuses attention on studies of the regulation of cdr2 expression. We have defined the tissue-specific expression pattern of the cdr2 antigen and found that it is regulated at a post-transcriptional level. A single cdr2 transcript is detected in nearly all tissues, whereas the PCD antigen is expressed specifically in brain and testis. Sequence analysis of both brain and spleen cdr2 cDNAs reveals that these mRNAs are identical, indicating that there is a tissue-specific regulatory mechanism responsible for restricting expression of the cdr2 protein that operates after mRNA processing.

The discrepancy between the distribution of cdr2 message and protein suggests several possible underlying mechanisms. Perhaps the most likely is that translational control regulates the expression of cdr2. Such a mechanism might relate either to an induction of translation specifically in brain and testis or a repression of translation in other tissues. There are several examples of tissue- or cell type-specific regulation of translation, including the testis proenkephalin mRNA, S-adenosylmethionine decarboxylase and



**Figure 7.** Analysis of *cdr2* expression by *in situ* hybridization (*A*, *C*, *E*) and immunohistochemistry (*B*, *D*, *F*). Sections of adult mouse brain (*A*, *B*), spleen (*C*, *D*), and testis (*E*, *F*) were hybridized with a  $^{33}\text{P}$ -labeled *cdr2* riboprobe or reacted with either affinity-purified or native PCD antisera. Dark-field photomicrographs reveal that the *cdr2* mRNA is detected in cerebellar Purkinje neurons, many brainstem neurons (*A*), splenic cortical cells (*C*), and cells of the outermost layers of the seminiferous tubules in the testis (*E*). There was no clear pattern of expression in the cerebral cortex, and no hybridization was observed with *cdr2* sense probes in any of the tissues examined (data not shown). Immunoreactivity with affinity-purified or native PCD antisera was detected in cerebellar Purkinje neurons, brainstem neurons (*B*, left and right panels, respectively), scattered neurons of the cerebral cortex (data not shown), and spermatogonia in the testis (*F*). Immunoreactivity was absent in spleen (*D*), which shows only background reactivity when compared with a normal human serum control (data not shown). *pc*, Purkinje cells; *gcl*, granule cell layer; *ml*, molecular layer; *io*, inferior olive; *rp*, red pulp; *ctx*, cortex; *spg*, spermatogonia; *spc*, spermatocytes.

the transcription factor BTEB (Hill and Morris, 1992; Rao and Howells, 1993; Imataka et al., 1994). Interestingly, the expression of BTEB closely resembles that of *cdr2*, in that the mRNA is detected in many tissues, whereas the protein is found only in brain and testis.

Most cases of translational regulation involve sequence elements in the 5' or 3'-UTRs of the mRNA. These elements may

form stable secondary structures that either directly impede the translation initiation complex or may act as binding sites for trans-acting regulatory factors (Meleforts and Hentze, 1993). In addition to complex secondary structure, many tightly regulated genes contain multiple upstream AUG codons (uAUGs), often present in long 5'-UTRs, that serve to decrease translational efficiency (Kozak, 1989, 1991a,b; Geballe and Morris, 1994). Both

S-adenosylmethionine decarboxylase and BTEB require the presence of such uAUGs in their 5'-UTRs for inhibition of translation. The 5'-UTR of human and mouse cdr2 cDNAs have ~80% G + C content in the 135 bp immediately upstream of the initiating methionine, predicting stable secondary structure. In addition, there are specific sequence elements within the cdr2 5'-UTR that are conserved across species.

Several alternate explanations for the discrepancy between the expression of cdr2 mRNA and protein may be considered. The cdr2 protein could be translated constitutively but selectively unstable because of a tissue-specific degradation mechanism. Although there are examples of proteins targeted for degradation in response to specific signals, there is little precedence for such a mechanism regionally restricting protein expression. Moreover, our observation that the cdr2 protein is able to be expressed at high levels when transfected into non-neuronal cells (data not shown) suggests that the stability of the protein is not likely to be dependent on tissue-specific factors.

It is also possible that our results reflect tissue-specific differences in post-translational modifications affecting the PCD epitope, such that the protein is only immunoreactive in brain and testis. Notably, the cdr2 leucine zipper harbors several potential phosphorylation sites. However, we have found that bacterially expressed cdr2 fusion protein, full-length cdr2 translated in reticulocyte lysate, and cdr2 protein expressed in a transfected fibroblast cell line are all readily detected by PCD antisera (Corradi and Darnell, unpublished observations). Therefore, it is unlikely that a neuron-specific post-translational modification, or lack thereof, is a significant factor in recognition of the cdr2 epitope. A more direct approach to address this question would be to generate antibodies against other epitopes of the cdr2 protein to examine expression of the antigen.

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## Review

# Onconeural antigens and the paraneoplastic neurologic disorders: At the intersection of cancer, immunity, and the brain

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**ABSTRACT** Paraneoplastic neurologic disorders (PNDs) are believed to be autoimmune neuronal degenerations that develop in some patients with systemic cancer. A series of genes encoding previously undiscovered neuronal proteins have been cloned using antiserum from PND patients. Identification of these onconeural antigens suggests a reclassification of the disorders into four groups: those in which neuromuscular junction proteins, nerve terminal/vesicle-associated proteins, neuronal RNA binding proteins, or neuronal signal-transduction proteins serve as target antigens. This review considers insights into basic neurobiology, tumor immunology, and autoimmune neuronal degeneration offered by the characterization of the onconeural antigens.

The unusual nature of a rare group of diseases, the paraneoplastic neurologic disorders (PNDs), offers the opportunity of developing insights into tumor immunology, autoimmune neurologic disease, and basic neurobiology. PNDs are believed to be autoimmune disorders that arise when systemic malignancies express proteins that are normally made only in neurons (onconeural antigens). A critical insight into the pathogenesis of the disorders was the recognition by Jerome Posner and coworkers that PND patients harbor high-titer antibodies in both their serum and spinal fluid that recognize apparently identical antigens in Western blots of normal brain and PND tumor tissues (refs. 1–5; for review see ref. 6). The presence of PND antibodies correlates with effective antitumor immunity (7–11), and their detection by Western blot both predicts the presence of specific underlying malignancies and definitively establishes the PND diagnosis (6, 12). Over the past several years PND antibodies have been used as reagents to clone and characterize a number of target PND antigens, allowing studies of a series of previously undiscovered neuronal proteins.

Antitumor immunity in PND patients became evident only by its association with severe neuronal degeneration. Most PND patients who present with neuronal degenerations are unaware that they harbor an occult malignancy (most commonly breast, ovarian, or small cell lung tumors); in rare instances, malignant neoplasms have been documented to vanish without treatment after the onset of neurologic disease (10, 13). Within the nervous system, nearly any group of neurons can be targeted in PND, including those of the limbic system, retina, cerebellum, brainstem, spinal cord, and dorsal root ganglia (Table 1). The immune system typically targets a single onconeural antigen in PND tumors, giving rise to a discrete set of neurologic symptoms [only very rarely have two distinct antigens been found to be targeted, leading to two superimposed neurologic syndromes (14–16)]. These observations suggest a model (considered below) in which the expression of neuronal antigens in tumor cells leads to an immune response which suppresses tumor growth but leads to the destruction of neurons (Fig. 1). It should be noted, however,

that this model rests solely on clinical data; to date, with the exception of the PNDs of the neuromuscular junction (NMJ), no animal model for these disorders has been established.

In addition to the significance that identification of the onconeural genes has for tumor immunology and autoimmune neurologic disease, these genes also encode proteins that are likely to be of unique importance to neurons. The model for the pathogenesis of the PNDs (Fig. 1) suggests that the normal expression of onconeural antigens is exquisitely restricted to immunologically privileged cells, allowing their recognition as foreign antigens when ectopically expressed in tumors. A corollary to this hypothesis is that as target antigens in neuronal degeneration, onconeural antigens are expressed in neurons; the data reviewed below suggest that in most cases onconeural genes are expressed exclusively in neurons. Thus the cloning of these genes using PND antisera provides an exceptional opportunity to study neuron-specific function. Finally, whereas the function of onconeural antigens is just beginning to be explored, the observation that specific tumor types express specific onconeural antigens (Table 1) suggests that the regulation of expression, and perhaps more importantly, the function of these proteins may have biologically important roles in tumor cells and neurons.

### Classification of the PNDs

The PNDs have traditionally been classified according to the clinical neurologic symptoms by which they were identified. Although recent work suggests that additional PNDs exist, four well-defined clinical syndromes initially led to the identification of four sets of antibodies and to the cloning of genes encoding target antigens (Table 1). These are (i) paraneoplastic cerebellar degeneration, in which women with breast or ovarian tumors harbor an antibody termed Yo that recognizes a 52-kDa antigen present in the tumors obtained from these patients as well as in cerebellar Purkinje neurons (6, 9, 17); (ii) paraneoplastic blindness (cancer-associated retinopathy), in which patients with small cell lung cancer harbor antibodies against a 23-kDa antigen present in the tumor cells and photoreceptors (18); (iii) paraneoplastic opsoclonus-myoclonus-ataxia (POMA), a motor disorder manifested by dysfunction of a subset of brainstem, spinal cord, and cerebellar neurons, in which patients with breast, fallopian, or lung tumors harbor an antibody that recognizes a 55-kDa antigen present in their tumor specimens and neuronal nuclei (19, 20); and (iv) paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/SN), a diffuse group of neurologic disorders manifested by symptoms of sensory loss, memory loss, cerebellar, brainstem, motor, or autonomic dysfunction that typi-

**Abbreviations:** PND, paraneoplastic neurologic disorder; NMJ, neuromuscular junction; POMA, paraneoplastic opsoclonus-myoclonus-ataxia; MG, myasthenia gravis; LEMS, Lambert-Eaton myasthenia syndrome; AChR, acetylcholine receptor; SMS, stiff-man syndrome; GAD, glutamic acid decarboxylase; n-RBP, neuron-specific RNA-binding protein; CNS, central nervous system; MHC, major histocompatibility complex.

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Table 1. PNDs defined by autoimmune antibodies

Neurologic syndrome			
Paraneoplastic name	Phenotype	Tumor	Antibody
Cerebellar degeneration Typical	Pan-cerebellar dysfunction (Purkinje cell)	Breast, ovarian	Yo
Atypical		None identified*	$\beta$ -NAP
Paraneoplastic opsoclonus myoclonus ataxia (POMA)	Motor (brainstem, spinal, cerebellar)	Breast, fallopian	Ri
Encephalomyelopathy/ sensory neuropathy (PEM/SN)	Multifocal Sensory Limbic Motor Autonomic Cerebellar	Small cell lung	Hu
Cancer-associated retinopathy	Blindness (photoreceptor)	Small cell lung	CAR
Stiff-man syndrome (SMS)	Motor (spinal interneuron?)	Breast	Amphiphysin
Paraneoplastic NMJ	Motor		
LEMS		Small cell lung	Various <sup>†</sup>
MG		thymoma	$\alpha$ -AChR

\*Expressed in neuroectodermal tumor lines.

<sup>†</sup>See text.

cally progress into a multisystem neuronal degeneration (14), in which patients with small cell lung cancer harbor an antibody termed Hu that recognizes 35- to 40-kDa antigens present in neuronal nuclei and small cell lung tumors (21). In addition to these paraneoplastic syndromes, two disorders involving the NMJ, myasthenia gravis (MG), and the Lambert-Eaton myasthenic syndrome (LEMS), are frequently associated with underlying malignancy, and are associated with antibodies to

the acetylcholine receptor (AChR) and the presynaptic calcium channel, respectively.

In this article I will review the current understanding of the nature of the onconeural antigens, reclassifying the PNDs into four discrete categories according to the nature of the target antigens (Table 2). A question that arises from this classification is whether proteins within a group share common features that render them susceptible to antineuronal immunity or that

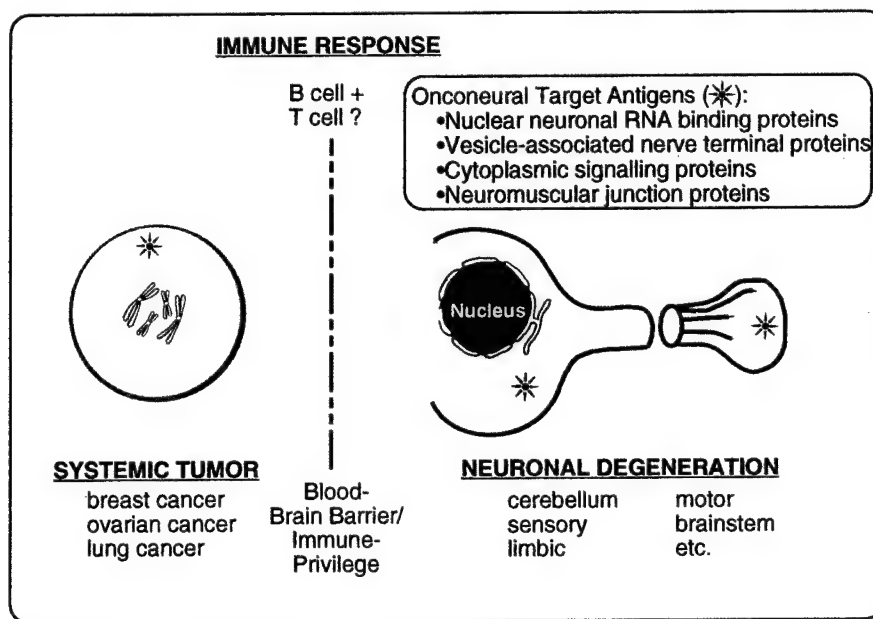


FIG. 1. Model for the pathogenesis of the PNDs. The paraneoplastic disorders are believed to initiate when solid tumors present outside of the nervous system express proteins that are normally made only in neurons. Perhaps in part because of the blood-brain barrier and/or the immunologically privileged state of neurons, the immune system recognizes the neuronal protein as foreign when ectopically expressed in tumor cells. A low level immune response is generated, which is associated with effective antitumor immunity. A second event is then postulated in which the ongoing antitumor immune response becomes competent to recognize neurons normally expressing the paraneoplastic antigen; such an event could either be disruption of the blood-brain barrier (e.g., cytokine mediated) or a change in the nature of the immune cells themselves. This establishes an autoimmune neurologic degeneration, which brings patients to clinical attention. There are two features of this model supported by data discussed in this review that are particularly relevant to neurobiology. (i) The model postulates that the effective immune recognition of neuronal antigens in tumor cells arises because the target antigens are normally exclusively expressed in neurons, both during development and during adulthood. If they were expressed outside of the immunologically privileged nervous system, they would be recognized as self-antigens and would not produce immunity. Thus, PNDs provide a means to study neuron-specific function through the identification of neuron-specific genes. (ii) The expression and function of the onconeural antigens, while not known, is of potential interest because specific tumor types selectively express specific neuronal proteins. This suggests that the regulation of expression and function of the onconeural genes may be particularly revealing to examine in tumor cells and neurons.



Table 2. Four categories of onconeural antigens

Antigen	Syndrome	Function
Nerve-terminal and vesicle-associated proteins		
Amphiphysin	SMS	Vesicle-associated; dynamin and AP2 interaction
$\beta$ -NAP	Cerebellar degeneration*	Vesicle coat protein (related to AP2)
Glutamic acid decarboxylase	SMS*	Synthesizes $\gamma$ -aminobutyric acid; vesicle associated
Synaptotagmin	LEMS	Vesicle-associated protein; AP2 interaction; $\text{Ca}^{2+}$ sensor
Neuron-specific RNA-binding proteins		
Nova family ( $\geq 2$ members)	POMA	KH motif RBPs; homologues to hnRNP-K, FMR-1, MER-1, PSI
Hu family ( $\geq 4$ members)	PEM/SN (multifocal)	RRM motif RBPs; mammalian homologues of elav
Putative neuronal signalling proteins		
cdr2 (Yo)	Cerebellar degeneration	Cytoplasmic protein; N-terminal amphipathic helix-leucine zipper
Recoverin	Blindness	cGMP-gated signal transduction
Neuromuscular junction proteins		
Presynaptic $\text{Ca}^{2+}$ channel ( $\beta$ -subunit)	LEMS	Signal transduction
AChR $\alpha$ -subunit	MG	Acetylcholine binding subunit of receptor

\*No tumor association.

correlate with their immunity in tumor cells. The cloning and characterization of onconeural antigens will allow for an examination of whether similarities in protein function, cellular location, antigenicity, susceptibility to functional disruption by antibody, or other features determine similar mechanisms of paraneoplastic neurologic disease. These questions are illustrated by considering each category of PND antigens individually.

### Neuromuscular Junction Antigens

Historically, the first paraneoplastic antigen identified was the  $\alpha$ -subunit of the nicotinic AChR, which is expressed at the postsynaptic motor endplate of the NMJ and is the target antigen in MG. Although MG is frequently thought of as an autoimmune neurologic disease, the AChR is expressed in thymomas associated with MG (22, 23), suggesting that in at least some cases MG is a paraneoplastic syndrome. In LEMS, a second PND involving the NMJ, patients harbor antibodies against components of the presynaptic motor endplate, and 60% of LEMS patients are found to have small cell lung cancer. *In vitro*, LEMS antibodies lead to impaired calcium flux (24, 25) and impaired acetylcholine release (26) from the presynaptic motor neuron. Unlike MG, a single autoantigen has not been identified in LEMS. Despite reports that many (27) or all (25) LEMS antisera bind to P/Q type voltage-gated presynaptic calcium channels, the specificity of such antibodies is uncertain, given that they can also be found in normal individuals or patients with unrelated neurologic disorders (25). A subset of LEMS patients harbor antibodies against other presynaptic nerve terminal components (see below), but the role of such antibodies in LEMS is uncertain.

MG and LEMS have several features that distinguish them from the other paraneoplastic disorders discussed here. First, the localization of the neurologic dysfunction at the NMJ places the site of immune attack at the border between neurons and the rest of the body, and the antigens under attack appear in extracellular localizations accessible to circulating antibodies. Second, autoantibodies play a demonstrated role in the pathogenesis of autoimmunity at the NMJ. For example, the major immunogenic epitope of the AChR is a region of the extracellular domain of the receptor susceptible to antibody attack (28, 29), and anti-AChR antibodies passively transfer the myasthenic syndrome to animals (28, 30). Although a

single dominant epitope has not been defined, mice treated with LEMS IgG have distorted active zones at the presynaptic junction (31) and LEMS antisera passively transfers the disease to animals (26, 32). Finally, both MG and LEMS patients, unlike other PND patients, benefit from plasmapheresis or immunosuppressive treatments that suppress B-cell function (33, 34).

In contrast to the NMJ target antigens, the PND antigens discussed below appear to be neuron-specific, intracellular proteins, and, where tested, the generation of PND antibodies in animals has failed to produce disease (35, 36). These observations suggest that paraneoplastic neurologic disorders involving the NMJ may have a fundamentally different pathophysiology from other PNDs. Whereas a mechanism of disease involving neuronal antibody uptake could be relevant for the remaining PNDs (see below), a role for other immune mechanisms (e.g., cytotoxic T lymphocytes or other cytolytic killer cell activity) seems particularly important to consider in these disorders, given their association with effective antitumor immunity and the intracellular localization of the target antigens.

### Nerve Terminal/Vesicle-Associated Antigens

Recent studies suggest that intracellular vesicle-associated proteins in the presynaptic nerve terminal form a distinct group of target antigens in both autoimmune and paraneoplastic neurologic disease. The first protein identified in this context is a target antigen in stiff-man syndrome (SMS; ref. 37), the enzyme glutamic acid decarboxylase (GAD), which converts glutamate to GABA. SMS is an autoimmune neurologic disorder (not generally associated with cancer) characterized clinically by motor symptoms consistent with GABAergic blockade, and the clinical symptoms are specifically ameliorated by GABA agonists. One mechanism for the pathogenesis of SMS consistent with these observations is that the autoantibody specific for GAD blocks the activity of this enzyme. GAD exists in the nerve terminal as a vesicle-associated soluble protein (38), suggesting that if antibody were taken up in the nerve terminal and able to gain access to the soluble proteins there, it might interfere with GAD protein function, much as antibodies microinjected into neurons *in vitro* are able to disrupt the function of vesicle membrane proteins in the nerve terminus (39).

Following the discovery of anti-GAD antibodies in SMS, a series of antibodies against intracellular presynaptic nerve

terminal proteins have been identified as autoantigens in neurologic disorders. A variant of SMS that develops in patients with breast cancer is associated with a paraneoplastic antibody against the nerve terminal/vesicle-associated protein amphiphysin (40). Amphiphysin appears to play a role in synaptic vesicle endocytosis in the nerve terminal by binding to the vesicle coat protein adaptor AP2 and dynamin (41, 42). Similarly, antiserum from a patient with an autoimmune cerebellar degeneration (in whom no tumor was identified) was used to characterize and identify a novel vesicle coat protein termed  $\beta$ -NAP (43, 44).  $\beta$ -NAP protein and mRNA are present exclusively in neurons where the protein exists as a cytoplasmic pool that appears to be recruited onto a subset of vesicles in both the cell soma and the nerve terminal (43, 107). Finally, in LEMS, a number of presynaptic nerve terminal proteins have been identified as antibody targets, including synaptotagmin (45, 46), a vesicle-associated calcium channel sensor, and an intracellular ( $\beta$ ) subunit of the presynaptic calcium channel (47).

Taken together, at least four vesicle-associated antigens present in the presynaptic nerve terminal have been clearly identified as autoimmune or paraneoplastic target antigens (Table 2). The common cellular localization of this set of antigens suggests that they may share a particular vulnerability to immunologic attack and/or disruption of function. It will be of interest to determine whether such selective vulnerability relates to a special susceptibility of nerve terminal proteins to inhibition of protein function following antibody exposure, or to other mechanisms such as selective antigen presentation and/or protein immunogenicity.

### Neuron-Specific RNA-Binding Proteins

A second discrete class of neuronal proteins to be identified as target antigens in PNDs are neuron-specific RNA-binding proteins (n-RBPs). Two distinct families of proteins fall into this category. The Nova antigens are homologous to a newly described class of RNA-binding proteins characterized by the presence of KH RNA-binding motifs (48). The Hu antigens comprise a second set of RNA-binding proteins characterized by the presence of three canonical  $\sim 80$  amino acid RNA recognition motifs (48). The Nova and Hu proteins share several common features: (i) they appear to be expressed exclusively in neurons from early in development through adulthood, (ii) they are predominantly expressed as nuclear proteins, and (iii) they harbor suggestive sequence homologies with RNA-binding proteins involved in the regulation of alternative splicing.

The Nova-1 gene was identified using high-titer antisera from a patient with POMA and breast cancer; serum reactivity with Nova-1 fusion protein is diagnostic for the disorder in adults, and serves to prompt physicians to search for the presence of occult breast, gynecologic, or lung tumors. Nova-1 expression is tightly restricted; the mRNA is only detectable in brain on Northern blots (19), and protein expression is restricted to the nucleus and, to a lesser degree, cytoplasm of neurons (49). Moreover, immunohistochemical and *in situ* hybridization studies reveal that Nova-1 expression is restricted to neurons of the central nervous system (CNS) throughout mouse development, where its expression is tightly restricted to the diencephalon, brainstem, and spinal cord (19, 49).

These observations are consistent with the paradigm of PND pathogenesis presented in this review; the restricted expression of the Nova antigen and mRNA to the CNS during development and into adulthood suggest that the protein is normally sequestered from the immune system and thereby potentially immunogenic in tumor cells. The distribution of Nova-1 mRNA within the brain correlates in an approximate way with the motor syndrome (involving an undefined mix of brainstem,

cerebellar, and/or spinal motor neurons) present in POMA patients (although these patients rarely develop a more diffuse encephalopathy; see ref. 50). This correlation has been complicated by the observation that POMA antisera used in immunohistochemical stains under nonstringent fixation conditions recognizes all CNS neurons (19, 20, 51); this discrepancy may partly be explained by the identification of at least one and perhaps as many as three additional Nova genes (ref. 19 and Y. Y. Yang, G. L. Yin, and R.B.D., unpublished observations) whose products are reactive with native POMA antisera. In addition, POMA antisera identifies a minor band of 70–80 kDa which has not been characterized. The specificity of neuronal dysfunction seen in POMA might result from differential susceptibility of either subsets of Nova antigens or subsets of Nova-expressing neurons to autoimmune attack.

Sequencing of the Nova-1 gene revealed the presence of three repeated motifs homologous with the repeated KH domains present in the hnRNP K protein (19). KH motifs are also found in FMR-1, the product of the fragile-X gene (52), and in two RNA-binding proteins implicated in the regulation of alternative splicing in *Drosophila* and yeast, termed PSI (53) and MER-1 (54), respectively. The Nova-1 protein has the characteristics of an RNA-binding protein *in vitro* where it binds to RNA with the same sequence preference (to ribohomoguanosine) as FMR-1. One hallmark of POMA disease antisera (found in six of six samples) is that they specifically recognize an epitope that lies within the third Nova-1 KH motif. Interestingly, affinity-purified POMA antibodies completely abrogate the RNA-binding activity of the intact Nova-1 protein (49). This *in vitro* observation is reminiscent of the suggestion made for SMS; that autoimmune antibodies may not only bind to but disrupt the function of their target antigens, and suggests that POMA antibody might act to disrupt the activity of the Nova-1 RNA-binding protein in neurons.

Antibodies to the Hu antigens are associated with a diverse set of neurologic degenerative disorders. Neuronal dysfunction localizes most commonly to the dorsal root ganglia (in  $\sim 60\%$  of Hu patients), but may relate to the cerebellum, brainstem, limbic system, motor neurons, or the autonomic nervous system, solely or as part of a multifocal disorder (14). Cloning of the Hu antigens using the patient's antisera originally yielded a single gene termed HuD that was found to encode a human homologue of the *Drosophila* elav protein (55). This connection was of importance because elav is a neuron-specific protein whose function is known to be essential for neurogenesis in *Drosophila* (56, 57), suggesting a potentially important role for HuD in mammalian neurobiology. Although no function has been determined for the Hu antigens, a target epitope in HuD, mapped using Hu disease antisera, localizes to the first two HuD RNA-binding domains (58). This suggests a possibility raised with the Nova antigens that antibody mediated disruption of the Hu RNA-binding activity might lead to neuronal death in patients with the neurologic syndrome.

Database alignments reveal that HuD is also highly homologous to the *Drosophila* RBP *sex-lethal*, primarily within the conserved RNA recognition motifs, but also to a significant degree in the sequence between them (55). This suggested a possibility that still has not been tested—that the Hu proteins may be involved in regulating alternative splicing within neurons—and spurred the cloning of additional family members by degenerate PCR and cDNA cloning. A total of four independent but highly homologous Hu genes have been identified that encode epitopes reactive with Hu antisera. Each of the Hu genes encode highly related n-RBPs, and each are alternatively spliced within their coding region.

Whether regional differences in the expression pattern of individual Hu genes or their spliced products correlate with the diverse neurologic symptomatology found in the Hu syndrome

is unknown. Immunohistochemical analysis (59) and *in situ* hybridization (60) performed with a probe from a conserved region of the Hu coding sequence demonstrate that Hu expression is restricted to neurons. *In situ* hybridization using gene-specific probes suggests marked variability in the developmental and tissue distribution of all four genes, in addition to confirming neuron-specific expression (H. J. Okano and R.B.D., unpublished data). Most patients with neurologic disease affecting predominantly one region of the nervous system ultimately develop a multifocal neurologic illness, dying from their neurologic disease an average of 7 months after the onset of symptoms. Thus, if the Hu autoimmune attack is initially directed to a single Hu gene product, it is possible that it ultimately becomes competent to recognize additional Hu family members.

In addition to the original HuD clone, the Hel-N1 (61), HuC (55, 62), and HuE (H. J. Okano and R.B.D., unpublished data) genes all encode members of the Hu family. Mapping of the mouse homologues of these four Hu genes reveals that they are clustered in pairs on two chromosomes, suggesting that they arose by gene duplication from a single common precursor (C. F. Fletcher, Copeland, N. G., Jenkins, N. A. and R.B.D., unpublished data). Biochemically, Hel-N1 has been shown to be an RBP in *in vitro* assays where it is able to bind to short stretches of uridylates and to AU-rich elements found in the 3' untranslated regions of some mRNAs (60, 61). However, the *in vivo* significance of these observations is unclear, in part because the biologic significance of the AU-rich sequences found to bind Hel-N1 is uncertain (63, 64), and in part because AU-rich elements are involved in many aspects of RNA metabolism, including the regulation of splicing and mRNA stability (65).

Given the extensive homologies between RNA-binding proteins that regulate alternative splicing and the Nova and Hu n-RBPs, it is tempting to speculate that these n-RBPs regulate alternative splicing in neurons, although a role for the proteins in regulating neuronal mRNA stability, translation, or subcellular localization remains both possible and of great potential interest. In considering the possibility that n-RBPs regulate neuronal splicing, it is worthwhile recalling the role of *sxl* in development (for reviews, see ref. 66 and 67). During the sexual development of flies, the *sxl* protein acts as a binary switch; in the presence of *sxl* females develop, in the absence of *sxl* males develop. The mechanism of *sxl* action is through its role as a sequence-specific RNA-binding protein; by binding to a polypyrimidine tract upstream of exon 3 of the *tra* primary transcript, *sxl* displaces the constitutive splicing machinery (specifically, the RNA-binding protein U2AF; ref. 68), forcing U2AF to bind to a secondary polypyrimidine tract, leading to usage of an alternative splice acceptor and a *tra* transcript that encodes a functional protein. Ultimately, a series of alternative splice choices becomes established, resulting in the development of female flies. Since the discovery of neuron-specific and nonneuronal spliced forms of the calcitonin-CGRP primary transcript (69, 70), there has been speculation that neuron-specific RBPs mediate fundamentally different exon usage in neurons.

The identification of the onconeural n-RBPs is consistent with such speculation, and their complexity extends it in several ways. The onconeural n-RBPs include at least six and possibly more members, present in two different gene families; moreover, there is extensive alternative splicing within the coding regions of the n-RBP genes themselves (19, 55, 71), with the potential to generate  $\geq 70$  n-RBP protein variants. The expression patterns of individual genes suggests specificity of expression within individual sets of neurons (R. Buckanovich, Y. Y. Yang, H. J. Okano, and R.B.D., unpublished data). The clearest example to date is Nova-1, whose mRNA is expression is tightly restricted to subsets of neurons; it is absent from the neocortex and thalamus, and abundant in regions of the

diencephalon, midbrain, and hindbrain (49). These observations suggest a role for n-RBPs that goes beyond the neuron-versus nonneuron binary switch suggested from the alternative splicing of calcitonin-CGRP, to a role in establishing unique characteristics of specific subsets of neurons. Since n-RBPs are targeted in adult neurologic syndromes, region-specific n-RBPs are also likely to be critical for the maintenance or function of sets of adult neurons. An attractive feature of the hypothesis that n-RBPs regulate splicing in neurons is that it suggests a means for the generation of diversity of neuronal function, using sets of n-RBPs to generate complexity from a limited size genome.

### Neuronal Signal Transduction Proteins

A final set of onconeural antigens includes a group of two proteins with potential roles in signal transduction pathways. The first such protein identified was the paraneoplastic retinal degeneration antigen recoverin. Antisera from patients who became blind in the setting of small cell lung cancer were used to characterize and ultimately clone the gene encoding a 23-kDa antigen expressed in photoreceptors. This clone turned out to encode recoverin (72, 73), although it should be noted that other uncharacterized paraneoplastic retinal antigens may also exist (18). The identification of recoverin as an onconeural antigen is of interest given the possible role of this protein in cGMP signal transduction cascade in photoreceptors. Disruption of this phototransduction signaling pathway leads to photoreceptor degeneration (74, 75), suggesting that targeted disruption of recoverin by the PND immune response may be involved in the photoreceptor degeneration seen in these patients.

The most common paraneoplastic cerebellar degeneration syndrome, described in 55 patients in ref. 9, occurs in patients with breast or ovarian cancer who harbor an autoantibody termed Yo, which has been used by two groups to clone a target antigen termed cdr2. Reverse-transcription (RT)-PCR analysis of a single tumor specimen from a Yo positive patient revealed expression of the cdr2 gene (J. P. Corradi and R.B.D., unpublished data), but not of cdr13, a minor antigen (on Western blots of Purkinje extracts) that was also cloned with Yo antisera (76). Immunity to the cdr2 antigen in gynecologic tumors is associated with effective antitumor immunity; 45/52 (87%) of patients with the Yo antibody and gynecologic cancer have limited tumors (9).

The cdr2 gene (77, 78) encodes a protein of predicted  $M_r$  of 52 kDa that harbors an extended amphipathic helix-leucine zipper domain in its N-terminal one-third (79), and a unique sequence of unknown function in its C-terminal two-thirds, suggesting separate dimerization and functional domains. The disease epitope has been mapped using Yo antisera, and localizes to the N-terminal leucine zipper domain (79). The Yo antigen localizes to the cell soma and cytoplasmic fractions of neurons, suggesting that it may interact with other leucine zipper proteins there. Identification of dimerization partners for cdr2, as well as a complete study of the expression pattern of protein, may yield insight into its role in both neurons and gynecologic tumors.

### Models of Disease Pathogenesis

The model for the pathogenesis of PNDs presented here (Fig. 1) has three essential features, which will be considered below: (i) onconeural antigens are normally expressed only in immune privileged sites and are immunogenic when ectopically expressed in tumors, (ii) antitumor immunity correlates with immunity to onconeural antigens, and (iii) antineuronal autoimmunity develops in a subset of patients with antitumor immunity.



**Immune Privilege of Onconeural Antigens.** Two components may be considered in establishing the immune privilege of onconeural antigens, one physical and one molecular. The blood-brain barrier is a physical barrier established, in part, by a specialized microvasculature and astrocytic foot processes that separates the CNS from the systemic circulation. This barrier can be breached by the immune system in disease (e.g., by cytokines such as IFN- $\gamma$  and TNF- $\alpha$ ), and may normally be "violated" by small numbers of immune cells that survey the CNS (80, 81). Thus, all individuals are likely to have some immune surveillance of the CNS by immune cells, and this mechanism is not likely to account for the strict immune privilege proposed for onconeural antigens. Moreover, some onconeural antigens (e.g., Hu antigens) are normally expressed in peripheral nervous system neurons (e.g., myenteric plexus neurons of the intestine) that lack a physical blood-brain barrier.

Molecular mechanisms of immune privilege are suggested by the observation that neurons do not normally appear to express major histocompatibility complex (MHC)-I molecules (82–84), or that testis, another immune privileged site, does express Fas (CD95) ligand (85). The absence of self-presentation molecules in neurons, or the induction of apoptosis of immune cells via Fas-like pathways (85, 86) provide mechanisms by which intracellular proteins (e.g., onconeural antigens) might entirely evade immune tolerance. Such a mechanism would be likely to be valid whether onconeural antigens are expressed in neurons of the gut, dorsal root ganglia, or CNS.

**Tumor Immunity in PND.** Immune privilege of onconeural antigens fails when the antigens are expressed in the tumors of PND patients. Two points broaden the scope of this observation. (i) While the neurologic complications found in the PNDs are rare (they complicate no more than 1 of 1000 cancer cases; see ref. 6), an unexpectedly large number of tumors are able to elicit immune responses to PND antigens. For example, 15% of small cell lung tumors are associated with low-titer antibodies to Hu antigens in the absence of neurologic disease, and these patients have a remarkably high percentage (>90%) of limited stage tumors (compared with small cell lung cancer patients who have no detectable Hu antibodies, 60–70% of whom have widely metastatic disease when diagnosed; see ref. 7). (ii) At least some PND antigens are expressed in a large percentage of tumors of a particular type. For example, the Hu antigens are expressed in all small cell lung tumors and most neuroblastomas (11, 87).

These observations suggest that ectopic expression of onconeural antigens may not be the sole determinant of their immunogenicity. Instead, in the course of evading immune surveillance, tumor cells may recapitulate some aspects of (molecular) immune privilege for certain onconeural antigens. Some support for this suggestion comes from evidence that a higher percentage of tumors associated with the Hu syndrome and antitumor immunity express MHC-I antigens than do tumors that are not associated with PND (11). Alternatively, host factors, such as specific MHC haplotypes, may act as determinants of whether an immune response is generated to onconeural antigens (or tumor cells), although no such correlation has been found.

The observed correlation between the development of detectable immunity to onconeural antigens and effective antitumor immunity does not suppose a cause and effect relationship between the two. Antitumor immunity, established independently from an immune response to onconeural antigens, might breach tumor immune privilege in such a way (e.g., by means of tumor cell apoptosis) that allows access to intracellular antigens and the secondary establishment of immunity to onconeural antigens. The proposal that immunogens such as RNA-binding proteins may be presented and targeted by the immune system in autoimmune disorders

following apoptotic events (88, 89) suggests that similar mechanisms might lead to immunogenicity of some onconeural antigens—for example, the Nova or Hu RNA-binding proteins. It should be noted, however, that PND antibodies are typically found in isolation, not in conjunction with other autoantibodies, including antinuclear antibodies.

The relationship between tumor immunity and immunity to onconeural antigens appears to differ for the various PNDs. In the NMJ paraneoplastic syndromes, where autoantibodies appear to be sufficient to mediate neurologic disease, there is no evidence to suggest that the presence of antibodies is associated with antitumor immunity. This correlation has only been established in PNDs involving intracellular antigens (most notably the Hu and Yo antigens), syndromes in which antibodies have failed to passively transfer disease. These observations suggest that, for some PNDs, antitumor immunity and immunity to onconeural antigens could be causally linked, and should heighten interest in whether cellular immunity plays a pivotal role in these disorders.

**Antineuronal Immunity in PND.** With the exception of the PND antigens of the NMJ, the PND antigens discussed in this review have different cellular distributions—nerve terminal, nuclear, or somato-dendritic/cytoplasmic—but are all intracellular proteins (Table 2). This observation presents difficulty for the hypothesis that antibodies mediate paraneoplastic neurologic disease. Nevertheless, a pathogenic role for PND antibodies in neuronal dysfunction cannot be entirely excluded. Various reports have suggested that some neurons may selectively take up macromolecules, including antibodies, into their cytoplasm (90–93). As noted above, antineuronal antibodies are able to passively transfer disease to animals in MG and LEMS, providing a compelling precedent for disease pathogenesis in the remaining PNDs. Finally, there are relatively higher titers of antibody in the CSF than serum (IgG index >1; refs. 6, 94, and 95), suggesting that there is an active B-cell inflammatory response within the CSF compartment of PND patients. Depending on the nature of the protein, antibody inhibition of function could be reversible or lead to neuronal death, and there is evidence for both types of neurologic dysfunction in PNDs. A significant number of POMA patients have complete resolution of their neurologic symptoms (96), suggesting the presence of dysfunctional but intact neurons in some patients. However, it should be noted that the most typical pathologic finding in PND is neuronal degeneration (6).

Autoimmune antibodies are believed to frequently target functional protein domains (97), and this appears to be true for PND antibodies. For example, the cdr2 epitope is the leucine zipper dimerization domain of the protein (79), the Hu epitope includes two RNA-binding domains (58), and the Nova-1 epitope is the third KH RNA-binding domain (49). In the latter case, PND antibodies inhibit the functional ability of Nova-1 to bind to RNA *in vitro* (49).

An additional means of antibody toxicity specific to neurons has been suggested by the observation that antibodies, and not T cells, may be responsible for eradicating latent viral infections in neurons. Antibodies to alphaviral proteins presented at the surface of latently infected neurons appear to restrict the expression of those proteins and eradicate viral infection, while cytotoxic T lymphocytes capable of recognizing the same antigens presented via MHC-I do not (98). If intracellular antigens are presented on the surface of neurons, an antibody mediated signal within the neuron may thus be able to inhibit antigen expression and thereby effect neuronal function.

The role of killer cells in the pathogenesis of PND has not been thoroughly explored. Several features of the PNDs make killer cells such as CD8<sup>+</sup> cytotoxic killer cells attractive candidates for mediators of disease. Most significantly, such cells have the potential to recognize intracellular onconeural antigens processed and presented via MHC-I molecules. An-

titumor immunity is thought to involve T-cell recognition of tumor antigens presented via MHC-I molecules (99, 100), in addition to other costimulatory signals; tumor immunity might set the stage for killer cell recognition of neurons. Moreover, the pathologic hallmark of PND is neuronal destruction (6, 101).

However, the lack of readily identifiable MHC expression on neurons (82) complicates the speculation that T cells are involved in the development of PNDs. While some studies have suggested that MHC molecules may be inducible in neurons *in vitro* (102), the significance of this observation *in vivo* is unclear. For example, transgenic mice made to express MHC-I molecules on neurons do not appear to undergo neuronal death following infection of neurons with lymphocytic choriomeningitis virus despite adoptive transfer of virus-reactive cytotoxic T lymphocytes (103). In summary, studies evaluating the pathogenesis of PND should consider the involvement of both classical and nonclassical T-cell types, including natural killer cells (which may recognize tumor or virally infected cells that fail to express MHC-I molecules; (104)),  $\gamma/\delta$  cells, and CD4<sup>+</sup>/CD8<sup>+</sup>  $\alpha/\beta$  cells that recognize antigens presented via MHC Ib and CD1 molecules (105, 106).

### Concluding Remarks

PNDs are a diverse group of diseases. Cloning target antigens using PND antisera allows both the clinician and scientist to discriminate between disorders that are otherwise similar in their symptomatology and pathology. In this way, an expanding group of onconeural antigens has been identified that shares common features—neuron-specificity and intracellular localization—but that can also be classified into several distinct groups based on their function. An important unresolved issue is whether the three identified roles of onconeural proteins—tumor antigens, autoimmune antigens, and neuron-specific proteins—are related. For example, specific tumor types consistently express specific onconeural antigens (Table 1). This selectivity is likely to yield clues to the role of onconeural antigens in PND. Individual onconeural genes could provide functions co-opted by tumor cells (as in the case of signal transduction proteins) or they might act as particularly potent immunogens (as in the case of n-RBPs) or be exposed in particularly vulnerable ways to the immune system (nerve terminal vesicle-associated proteins). Similarly, onconeural genes might be activated in trans with an activity that is directly selected by tumor cells (e.g., coordinate transcriptional controls with a cellular oncogene) or their expression might relate to the cell of origin of the tumor (e.g., small cell lung cancer as a neuroectodermally derived tumor). Perhaps the PNDs are best viewed as a diverse group of neurologic disorders, in which mechanisms of disease may share some common features but have different pathophysiologies that are likely to relate to different families of antigens. The ability to classify the target antigens based on the sequence and function of the proteins will be of value in establishing the pathophysiology for individual disorders.

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